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PROPRIEDADES QUÍMICAS E BIOLÓGICAS DE ÓLEOS ESSENCIAIS, EXTRATOS E FITOQUÍMICOS DE ESPÉCIES FRUTÍFERAS: ESTUDO PROSPECTIVO

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O que faz a gente ser grande é não perder o futuro de vista. É chegar a uma porta, fincar a bandeira da conquista e nesse mesmo instante começar a buscar outros portos. É criar desafios, calcular riscos, avançando sempre. Porque a grande aventura é viver. E a vida, assim como as ondas, tem um jeito diferente de se repetir. De prometer descobertas e abrigar todos os tipos de sonhos e embarcações. O que faz a gente ser grande é ser como o mar: incansável na sua procura pela onda perfeita. Até descobrir que a perfeição está na própria busca.

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ABTS	2,2-azinobis-3-etil-benzotiazolina-6-ácido sulfónico
Ar	arila
$Ar_2Se=O$	selenóxido
$ArSe^{\bullet+}$	radical cátion selenil
CAT	catalase
COX-2	cicloxigenase tipo 2
DHZ	deidrozingerona
$DHZO^-$	íon fenóxido da deidrozingerona
DPPH	2,2-difenil-1-picrilhidrazil
ERN	espécie(s) reativa(s) de nitrogênio
ERO	espécie(s) reativa(s) de oxigênio
GPx	glutationa peroxidase
GR	glutationa redutase
GSH	glutationa reduzida
GSSG	glutationa oxidada
GST	glutationa S-transferase
$L^\bullet$	radical livre lipídico
LDL	lipoproteínas de baixa densidade
LH	ácido graxo poli-insaturado
$LOO^\bullet$	radical peroxil
LOO^-	íon peroxil
LOOH	hidroperóxido
MDA	malondialdeído

NAD(P)H	nicotinamida adenina dinucleotídeo fosfato
NPS	nitroprussiato de sódio
SOD	superóxido dismutase

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Resumo

Martinez, Débora M.. **Propriedades químicas e biológicas de óleos essenciais, extratos e fitoquímicos de espécies frutíferas: Estudo prospectivo**. 2014. Tese (Doutorado) - Programa de Pós-Graduação em Ciência e Tecnologia Alimentos. Universidade Federal de Pelotas, Pelotas.

A principal abordagem deste trabalho foi o estudo prospectivo das propriedades químicas e bioquímicas de recursos naturais, incluindo o óleo essencial (OE) das folhas da *Campomanesia xanthocarpa*, os frutos das espécies nativas *Psidium cattleianum* e *Butia odorata* e o composto de ocorrência natural deidrozingerona (DHZ), presente no rizoma da *Zingiber officinale*. As capacidades antioxidante e antimicrobiana *in vitro* e a toxicidade aguda do OE das folhas de *C. xanthocarpa* foram determinadas. O OE apresentou significativa atividade protetora contra a peroxidação lipídica induzida do ácido linoleico e tecidual [fígado, rim, cérebro e estruturas cerebrais (hipocampo, córtex e cerebelo)] de camundongos. Esse potencial pode ser atribuído em parte aos compostos majoritários do OE (linalol e fenchol) e às suas propriedades quelante (Fe^{2+}) e de neutralização de radicais livres (DPPH e ABTS). A capacidade antibacteriana e antifúngica do OE foi significativa para cepas de importância clínica e em alimentos, dentre as quais: *Staphylococcus aureus*, *Salmonella typhimurium, Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Candida* spp, *Tripchosporon asahii* e *Cryptococcus laurentii*. O OE não apresentou toxicidade aguda quando administrado por via oral (100 e 500 mg/kg) em camundongos. Os frutos de *P. cattleianum* e *B. odoratta* (var. amarelo e vermelho) liofilizados apresentaram capacidade antioxidante pela diminuição da peroxidação lipídica induzida *in vitro* em estruturas cerebrais de camundongos (hipocampo, córtex e cerebelo). Os frutos de *P. cattleianum* apresentaram maior potencial antioxidante que os de *B. odorata*, principalmente na capacidade de neutralização de espécies altamente reativas, como o $^{\bullet}OH$ e radicais DPPH, e maior potencial antioxidante na peroxidação lipídica do hipocampo. O tratamento sub-crônico (35 dias) por via oral de camundongos com os frutos de *P. cattleianum* e *B. odorata* (var. amarela) (100, 500 e 1000 mg/kg) não alterou nenhum dos parâmetros de toxicidade avaliados no soro, tecidos hepático, renal e cerebral dos animais. Por outro lado, o tratamento com *B. odorata* diminuiu os níveis de peroxidação lipídica no rim (500 e 1000 mg/kg) e no fígado (100 mg/kg) dos animais. O consumo de *P. cattleianum* aumentou o potencial redutor do soro sanguíneo (100 mg/kg). Observou-se que a inserção de um grupo Se- ou S-aril na ligação *α-β*-insaturada ou de um grupo Se-aril ao carbono terminal da DHZ conferiram atividade antioxidante em sistema de hemólise induzida por 2,2'-azo(2-asmidinopropano)diidrocloreto (AAPH) e na peroxidação lipídica do ácido linoleico. Além disso, nos adutos de Michael contendo selênio e enxofre, este efeito antioxidante pode ser explicado devido ao maior potencial de neutralização de radicais livres (DPPH). Os resultados demonstraram o significativo potencial biológico de novos compostos organocalcogênios derivados da DHZ, do OE de *C. xanthocarpa* e dos frutos de *P. cattleianum* e *B. odorata*, apontando para alternativas viáveis para prospecção de novos agentes antioxidantes.

Palavras-chave: *Psidium cattleianum*, *Butia odorata*, *Campomanesia xanthocarpa,* organocalcogênios, deidrozingerona.

Abstract

Martinez, Débora M.. **Biological and chemical properties of essential oils, extracts and phytochemicals of fruit species: Prospective study**. 2014. Thesis (PhD) – Graduate Program in Food Science and Technology. Federal University of Pelotas, Pelotas.

The main approach of this work was a prospective study of chemical and biochemical properties of natural resource, including essential oil (EO) of *Campomanesia xanthocarpa* leaves, fruits of *Psidium cattleianum* and *Butia odorata* species and the naturally occurring compound dehydrozingerone*,* present in *Zingiber officinale* rhizome. The *in vitro* antioxidant and antimicrobial capacities and acute toxicity of EO of *C. xanthocarpa* leaves were determined. The EO presented significant protective activity against the linoleic acid and tissues [liver, kidney, brain and brain structures ((hippocampus, cortex, cerebellum)] induced lipid peroxidation of mice. This potential can be attributed in part to the major compounds (linalool and fenchol) and to its chelating property (Fe^{2+}) and free radicals scavanging (DPPH and ABTS). The antibacterial and antifungal capacity of EO was significant for clinical and food important strains, such as: *Staphylococcus aureus*, *Salmonella typhimurium, Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Candida* spp, *Tripchosporon asahii* and *Cryptococcus laurentii*. The EO didn't present acute toxicity when administred orally (100 and 500 mg/kg) in mice. *P. cattleianum* and *B. odoratta* lyophilized fruits (yellow and red var.) showed antioxidant capacity by decrease of induced lipid peroxidation of brain structures of mice (hippocampus, cortex and cerebellum). *P. cattleianum* fruits showed higher antioxidant potential than those *B. odoratta*, mainly on ability to neutralize highly reactive species such as $^{\bullet}OH$ and DPPH, and higher antioxidant potential on lipid peroxidation of hippocampus. The sub-chronic treatment (35 days) by oral route of mice with *P. cattleianum* and *B. odoratta* (yellow var.) lyophilized fruits (100, 500, 1000 mg/kg) didn't alter any of toxicity parameters evaluated in serum, liver, kidney and brain tissues. On the other hand, the treatment with *B. odorata* decreased lipid peroxidation levels on kidney (500 and 1000 mg/kg) and liver (100 mg/kg). *P. cattleianum* intake increased the serum potential reduction (100 mg/kg). It was observed that the insertion of Se- or S-aryl group in the *α-β*-unsaturated bond or a Se-aryl group in the terminal carbon of DHZ have given antioxidant activity on hemolysis system induced by 2,2′-azo(2-asmidinopropane)dihydrochloride (AAPH) and linoleic acid lipid peroxidation. Furthermore, on the Michael adducts containing selenium and sulfur, the antioxidant effect can be explained by the greater potential for neutralizing free radicals (DPPH). The results demonstrated the significant biological potential of new organochalcogen compounds derived from oDHZ, EO of *C. xanthocarpa* and *P. cattleianum* and *B. odorata* fruits, pointing to viable alternatives to prospection of new antioxidants agents.

Keywords: *Psidium cattleianum*, *Butia odorata*, *Campomanesia xanthocarpa*, organochalcogen, dehydrozingerone.

1 Introdução

O estresse oxidativo é um estado caracterizado pelo desequilíbrio entre a produção de espécies reativas e o sistema de defesas antioxidantes do organismo, a favor dos oxidantes (SIES, 1997). Os efeitos do estresse oxidativo abrangem desde respostas adaptativas até danos irreversíveis a constituintes vitais, como as proteínas, os lipídios e o DNA (HALLIWELL, 2005).

Há evidências de que o estresse oxidativo está envolvido na patofisiologia de doenças crônicas como diabetes, doenças cardiovasculares (aterosclerose, hipertensão, hipertrofia cardíaca), doenças neurogenerativas (doença de Parkinson, doença de Alzheimer e doença de Huntington), perda de memória e depressão (MARTÍNEZ, 2006; BROWNE e BEAL, 2006; MAES et al., 2011; RAMAMOORTHY et al., 2012; RAJENDRAN et al., 2014).

O organismo apresenta um sistema antioxidante constituído de defesas enzimáticas e não enzimátivas endógenas, capazes de manter o estado redox do organismo. Além destas, há as defesas antioxidantes exógenas, das quais destacam-se vitaminas (C e E), elementos minerais (Se) e fitoquímicos (compostos fenólicos, carotenóides, flavonóides) proveninentes da dieta. Alguns estudos revelam que estes componentes podem auxiliar na diminuição da incidência de doenças degenerativas (FESKANICH et al., 2000; GORDON, 1996; HALLIWELL, 1996). Neste sentido, o consumo frequente de frutas e hortaliças vem sendo associado à melhoria de condições cardiovasculares e neurológicas, redução da incidência de câncer e aumento da longevidade (BOEING et al., 2012; TRAKA e MITHEN, 2011).

Os frutos das espécies nativas *Psidium cattleianum* (araçá), *Butia odorata* (butiá) e *Campomanesia xanthocarpa* (guabiroba) apresentam fitoquímicos com potencial exploração como compostos funcionais. Dentre estes, destacam-se a vitaminha C, os compostos fenólicos, os carotenóides e as antocianinas (MEDINA et al., 2011; PEREIRA et al. 2012; FARIA et al., 2011; LOPES, et al 2011). Neste sentido, extratos orgânicos de araçá apresentam capacidade antioxidante, antiproliferativa de células cancerígenas (LUXIMAN-RAMMA et al., 2003; FETER et al., 2010; MEDINA et al., 2011) e anti-inflamatória (MCCOOK-RUSSEL et al., 2012). Os teores de carotenóides e vitamina C do butiá, ganharam destaque em estudo que

também indicou seu potencial antioxidante *in vitro* (PEREIRA et al., 2013; FARIA et al., 2011). Além disso, a atividade pró-vitamina A do butiá é significativa, tanto que o fruto é capaz de suprir as necessidades dietéticas desta vitamina (IOM, 2001).

Embora os frutos da guabirobeira, apresentem elevados níveis de vitamina C e carotenóides, são escassos dados sobre suas propriedades biológicas (PEREIRA et al., 2012). Por outro lado, os extratos obtidos a partir das folhas desta espécie têm maior investigação científica e têm a capacidade de diminuir os níveis de colesterol e glicose em condições de hiperlipidemia e diabetes (VINAGRE et al., 2010; KLAFKE et al., 2012).

Dentre os compostos bioativos de ocorrência natural, a deidrozingerona (DHZ) é uma hidroxifenilbutenona presente no gengibre (*Zingiber officinale*). O gengibre é uma raíz comestível utilizada como ingrediente e aromatizante em alimentos e cosméticos (SINGH et al., 2014). A DHZ, possui um largo espectro de atividades biológicas, sendo algumas correlacionadas com a atividade antioxidante (PRIYADARSINI et al., 1998; KANCHEVA et al., 2013), como a capacidade radioprotetora, antimutagênica (PARIHAR et al., 2007) e anti-inflamatória (SALDANHA et al., 1990). Além do aspecto biológico, o interesse do estudo da DHZ deve-se a presença de sítios reativos e possível modificação estrutural. Neste sentido, a inclusão de grupos funcionais pode conferir ou potencializar sua atividade biológica (KUBAR et al., 2014). Esta perspectiva, motivou o estudo da inserção de grupos orgânicos contendo calcogênios (Se e S) a fim de potencializar a propriedade antioxidante da DHZ.

Neste contexto, compostos organocalcogênios, com destaque aos que contêm Se e S em sua estrutura, são reconhecidos por diversas propriedades farmacológicas. Os compostos orgânicos de selênio, possuem atividade antioxidante, neuroprotetora (ROSSATO et al., 2002), antidepressiva (BORTOLATTO et al., 2012), antinociceptivo, anti-inflamatória (SAVEGNAGNO et al. 2007), anti-úlcera (SAVEGNAGO et al., 2006). Além disso, a capacidade de compostos orgânicos de Se em melhorar a memória e o aprendizado (CECHELLA et al., 2014), reverter sintomas da doença de Alzheimer (PINTON et al., 2013) e inibir convulsões já foram relatados em modelos experimentais *in vivo* (WILHELM, et al., 2009; 2010).

Considerando que o potencial antioxidante e a toxicidade de espécies frutíferas nativas ainda são pouco explorados, os estudos compreendidos neste trabalho possuem caráter exploratório sobre estes aspectos em modelos experimentais *in vitro* e *ex vivo* dos frutos araçá e butiá. Buscou-se também avaliar o potencial biológico do óleo essencial das folhas da espécie *Campomanesia xanthocarpa* quanto à capacidade antioxidante, antimicrobiana e toxicidade. No contexto da bioatividade de compostos orgânicos naturais e organocalcogênios, este trabalho apresenta pela primeira vez, a síntese e a atividade antioxidante *in vitro* de compostos contendo selênio e enxofre análogos à DHZ.

1.1 Hipóteses

a) A modificação estrutural da molécula deidrozingerona pela adição de grupos orgânicos contendo selênio e enxofre potencializa sua atividade antioxidante *in vitro.*

b) O óleo essencial das folhas de *Campomanesia xanthocarpa* apresenta atividade antioxidante e antimicrobiana e ausência de toxicidade aguda.

c) Os frutos de *Psidium cattleianum* e *Butia odorata* liofilizados possuem capacidade antioxidante *in vitro* e *ex vivo* e ausência de toxicidade *ex vivo*.

1.2 Objetivos

a) Sintetizar compostos organocalcogênios análogos à deidrozingerona e avaliar a atividade antioxidante *in vitro* dos compostos.

b) Determinar a composição química, a atividade antioxidante *in vitro* e toxicidade aguda do óleo essencial das folhas de *C. xanthocarpa* em camundongos.

c) Avaliar a capacidade antioxidante *in vitro* do araçá (*P. cattleianum*) e butiá (*B. odorata*) e seus efeitos em um tratamento sub-crônico avaliando parâmetros bioquímicos e toxicológicos em camundongos.

1.3 Estrutura da tese

A tese está estruturada, a partir da Introdução, em seis seções: i) referencial teórico geral. ii) manuscrito científico sobre a síntese e atividade antioxidante *in vitro* de novos compostos organocalcogênios análogos à deidrozingerona; iii) manuscrito científico da pesquisa exploratória sobre a capacidade antioxidante, antimicrobiana e toxicidade do óleo essencial das folhas de *C. xanthocarpa* (guabiroba); iv) manuscrito científico da pesquisa exploratória sobre a capacidade antioxidante *in vitro* de araçá (*P. cattleianum*) e butiá liofilizados (*B. odorata*); v) manuscrito científico sobre o estudo da capacidade antioxidante, toxicidade e genotoxicidade *ex vivo* de araçá e butiá liofilizados; vi) considerações finais e perspectivas.

2 Revisão da Literatura

2.1 Espécies reativas, estresse oxidativo e defesas antioxidantes

Durante o metabolismo aeróbio e processos de transferência de energia normais nas células, mais especificamente na cadeia transportadora de elétrons localizada nas mitocôndrias, ocorre a redução do oxigênio molecular (O_2) à água. No entanto, uma pequena fração do O_2 (0,1-0,5%) sofre redução com a geração do radical ânion superóxido ($O_2^{\bullet-}$) e em seguida a redução à peróxido de hidrogênio (H_2O_2). Além disso, durante a fosforilação oxidativa, 1-3% de elétrons são perdidos nos complexos respiratórios I e III da cadeia transportadora de elétrons que dão origem ao $O_2^{\bullet-}$ (DELATTRE et al., 2005; VALKO et al., 2007) (Figura 1). O $O_2^{\bullet-}$ também é gerado pela enzima xantina oxidase, que tem papel fisiológico sobre o metabolismo das purinas. Outros processos geram esta espécie reativa, como a ação da NAD(P)H oxidase (em processos oxidativos estimulados por fagocitose) e do Citocromo P450 (no metabolismo de fase I) (BOUAYED, 2010).

A geração de $O_2^{\bullet-}$ é uma das principais vias de formação de espécies reativas de oxigênio (ERO) como H_2O_2, radical hidroxil ($^{\bullet}OH$), radical alcoxil ($LO^{\bullet}$), e radicais peróxil ($LOO^{\bullet}$) (BOUAYED, 2010).

As ERO geradas possuem alta reatividade, como exemplo, o H_2O_2 é capaz de gerar o $^{\bullet}OH$, a partir da reação com íons metálicos através das reações de de Fenton, ou com o $O_2^{\bullet-}$ na reação de Haber-Weiss (RADI, 1993) (Figura 2).

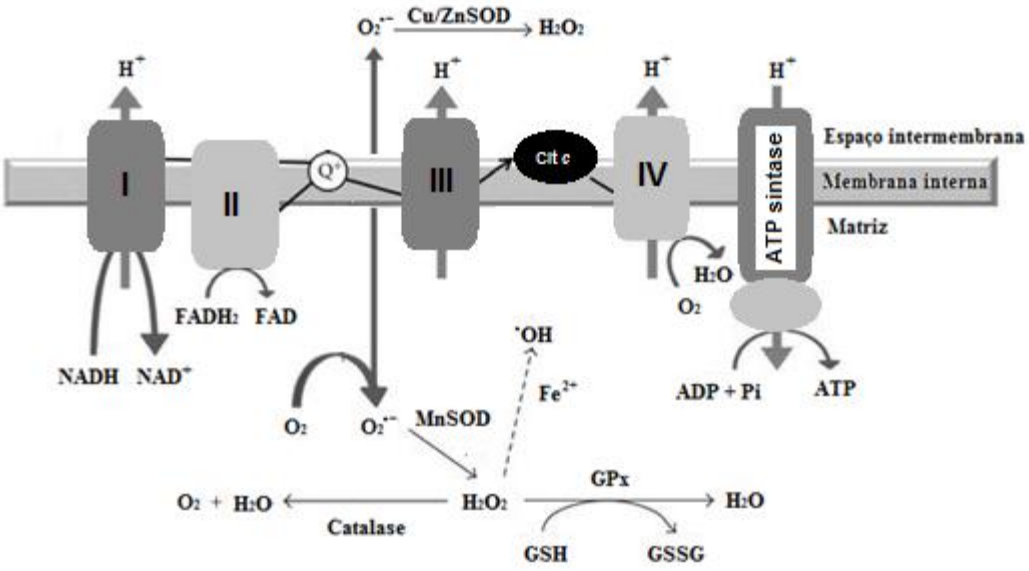


Figura 1 – Esquema geral da cadeira transportadora de elétrons com a formação e transformação de espécies reativas.

Algumas espécies reativas (ER) são classificadas como espécies reativas de nitrogênio (ERN), como exemplo tem-se o óxido nítrico ($^{\bullet}NO$). O $^{\bullet}NO$ é um mensageiro nas funções no sistema nervoso central (neurotrasmissor) e no sistema circulatório (vasodilatador). No entanto, o $^{\bullet}NO$ participa da geração de outras ER, quando reage com o excesso de $O_2^{\bullet-}$ e forma o peroxinitrito ($ONOO^-$). O $ONOO^-$ é uma espécie não-radicalar fortemente oxidante, podendo oxidar proteínas mitocondriais e os lipídios localizados nesta estrutura (BECKMAN, 1996; CAMARA et al., 2010). Em geral, a reatividade do $ONOO^-$ se dá por sua decomposição em dióxido de nitrogênio ($NO_2^{\bullet}$) e $^{\bullet}OH$, além de também reagir com dióxido de carbono (CO_2) formando carbonato ($CO_3^{\bullet-}$) e $NO_2^{\bullet}$ (SOUZA et al., 2008).

O $^{\bullet}NO$ e suas ER derivadas, são conhecidos oxidantes biológicos envolvidos nos principais processos fisiológicos e patofisiológicos como a inflamação, apoptose, regulação da atividade enzimática e expressão gênica, através da modificação oxidativa de lipídios, proteínas e DNA (RADI et al., 1991, 2001; RADI, 2004).

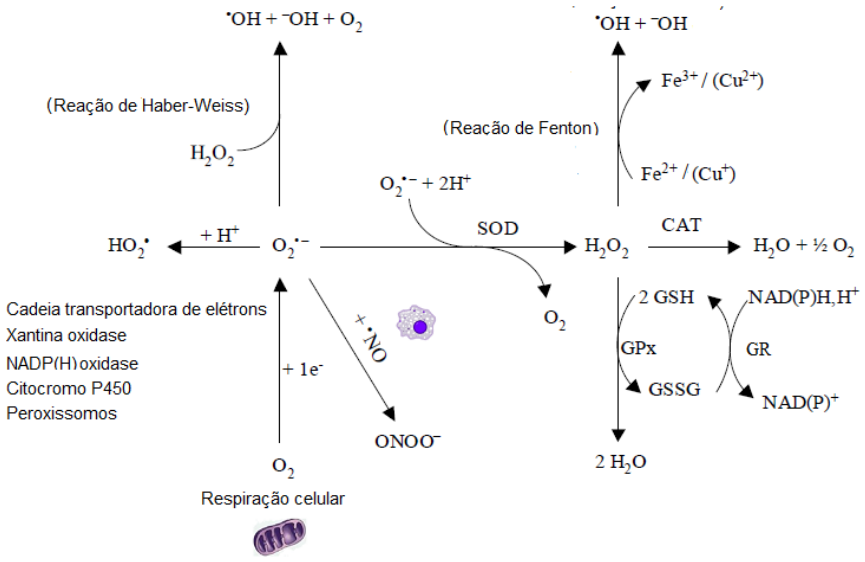


Figura 2 – Principais vias de formação e transformação de ERO/ERN. CAT: catalase; SOD, superóxido dismutase; GPx: glutationa peroxidase; GR: glutationa redutase; GSH: glutationa; GSSG: glutationa oxidada; $NAD(P)^+$ e NAD(P)H: formas oxidada e reduzida da nicotinamida adenina dinucleotídeo fosfato, respectivamente.

Resumidamente, a maior parte das ER são produzidas pelas células a partir de diferentes vias, como:

(a) a respiração aeróbia normal na mitocôndria, onde é gerado o $O_2^{\bullet-}$ e produtos mais reativos, como $^{\bullet}OH$;

(b) macrófagos estimulados e leucócitos polimorfonucleares, que liberam $O_2^{\bullet-}$ e $^{\bullet}NO$, os quais interagem para a formação de $ONOO^-$;

(c) peroxissomos, organelas celulares que produzem H_2O_2 como produto da degradação de ácidos graxos e outras moléculas; e

(d) a partir de produtos da oxidação que ocorre durante a indução das enzimas do citocromo P450.

As ER, quando em concentrações elevadas, causam um desequilíbrio redox celular frequentemente associado a causa e/ou progressão de doenças crônicas, degenerativas e ao envelhecimento. Isto deve-se aos consequentes danos oxidativos em componentes da estrutura celular causados pelo estresse oxidativo (HALLIWELL e GUTTERIDGE, 1999; SOHAL el al., 2002; VALKO et al., 2007).

Mais especificamente, o estresse oxidativo e nitrosativo portanto, é caracterizado pelo desequilíbrio entre a formação ou concentração de ER e as defesas antioxidantes. Esta condição é responsável pela ocorrência de danos permanentes em lipídios (O'DONNELL et al., 1999; RUBBO et al., 1994), em proteínas e nas bases do DNA (CAULFIELD et al., 1998; AKAIKE et al., 2003). Por isso esta condição é envolvida na indução de respostas auto-imunes prejudiciais e facilitam a ocorrência de falhas no funcionamento celular normal. Há também evidências de que o estresse oxidativo está envolvido em doenças neurodegenerativas, principalmente Alzheimer e Parkinson (VALKO et al., 2007).

Os seres vivos possuem um sistema de defesa antioxidante celular desenvolvido que atuam de modo a evitar o acúmulo de ER e manter o equilíbrio redox celular. Este sistema é composto por defesas enzimáticas e não enzimáticas. As principais defesas antioxidantes enzimáticas compreendem as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa S-transferase (GST); e as defesas não-enzimáticas a glutationa, ascorbato, tocoferóis, carotenóides, entre outros (WANG et al., 2009; HALLIWELL e GUTTERIDGE, 2007).

A SOD catalisa a dismutação do $O_2^{\bullet -}$ em H_2O_2, o qual é transformado em água tanto pela CAT quanto pela GPx. A ação da GPx se extende à redução de peróxidos (ROOH), com o requerimento de glutationa (GSH) como co-fator doador de elétrons. Assim, o ciclo catalítico da GPx é mantido pela glutationa redutase (GR) que converte a glutationa oxidada (GSSG) à sua forma reduzida (Figura 2). A enzima GST desempenha a função no metabolismo de xenobióticos, catalisando sua conjugação com a GSH para facilitar o processo de excreção. Esta enzima possui atividade antioxidante devido a capacidade de neutralizar hidroperóxidos orgânicos (HALLIWELL e GUTTERIDGE, 2007).

A GSH (γ-L-glutamina-L-cisteinil glicina), é o tiol não protéico mais abundante nas células de mamíferos, desempenha um importante papel na detoxificação de xenobióticos, atua como co-fator das enzimas GPx e GST, e aprensenta ação antioxidante *per se* sobre ER (BRAY e TAYLOR, 1993). Além da GSH, compõem o sistema antioxidante não-enzimáto o ácido úrico, NADPH, coenzima Q, albumina e a bilirrubina (BOUAYED e BOHN, 2012).

2.2 Peroxidação lipídica

A lipoperoxidação ou peroxidação lipídica (PL) é qualquer reação de degradação oxidativa de lipídios, sendo uma importante variável no estudo de condições biológicas relacionadas ao estresse oxidativo (HALLIWEL e GUTERIDGE, 2007). Assim, a PL tem recebido atenção do ponto de vista químico, bioquímico, nutricional e medicinal. Os lipídios, como outras biomoléculas (proteínas, carboidratos e ácidos nucléicos) e principalmente ácidos graxos poli-insaturados, são alvos de ER. O processo de PL é constituído de três etapas principais: iniciação, propagação e términação. A etapa de iniciação é dada pela ação de pró-oxidantes que abstraem um átomo de H de um ácido graxo poli-insaturado (LH) gerando um radical livre lipídico ($L^{\bullet}$). Na etapa de propagação, este radical reage com o oxigênio molecular, gerando os radicais peroxil ($LOO^{\bullet}$) que oxidam outras moléculas de ácidos graxos. Assim gera-se novos radicais lipídicos e hidroperóxidos (LOOH). Na fase de término, a decomposição destes intermediários gera outros produtos, e é também onde a ação de um antioxidante, como o *α*-tocoferol, é efetiva (Figura 3). De modo geral, os danos oxidativos aos lipídios levam rapidamente à alteração da integridade, fluidez e permeabilidade de biomembranas; a disfunção de receptores e degradação de lipoproteínas de baixa densidade (LDL) e colesterol (GREENBERG et al., 2008). Além disso, há vários produtos finais do processo de PL, gerados a partir da decomposição de hidroperóxidos, como os aldeídos eletrofílicos altamente reativos malondialdeídos (MDA), 4-hidroxi-2-nonenal (4,2-HNE) e acroleína (PORTER, 1986; ESTERBAUER et al., 1991; LOIDL-STAHLHOFEN et al., 1994). O MDA é o produtos mais mutagênicos, enquanto o 4,2-HNE o mais tóxico gerado na PL (ESTERBAUER et al., 1990; WEST et al., 2006).

Mais especificamente, o MDA é formado pela decomposição de peróxidos bicíclicos derivados de ciclização de hidroperóxidos. Em processos enzimáticos, como a biossíntese de tromboxano A_2, o MDA é formado como produto secundário (HECKER e ULLRICH, 1989).

Um aspecto importante a ser salientado, é que pouco se conhece sobre o papel fisiológico do MDA, uma vez que níveis elevados são encontrados em fluidos biológicos em situações de estresse oxidativo. O MDA possui alta reatividade com proteínas e DNA levando a formação de adutos, os quais podem participar de

reações deletérias secundárias pela formação de ligações cruzadas proteína/DNA. Estes efeitos podem induzir significativamente a ocorrência de alterações funcionais dessas biomoléculas (AYALA et al., 2014).

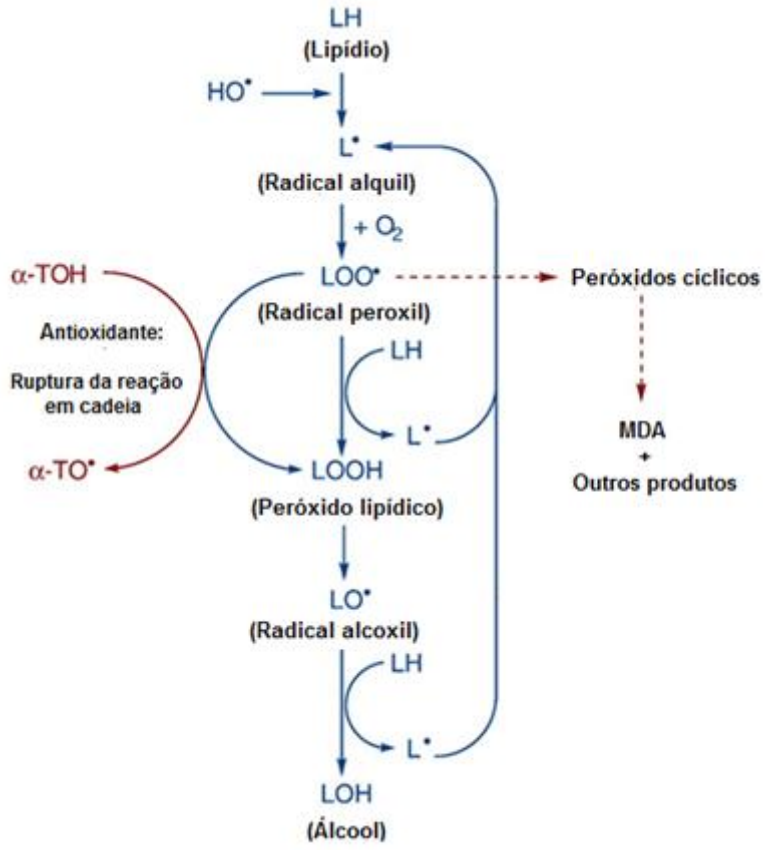


Figura 3 – Etapas, intermediários e produtos da peroxidação lipídica. Fonte: Sachdev e Davies (2008).

Com base nesta fundamentação, neste trabalho, foram avaliados os níveis de PL em diferentes sistemas *in vitro* e *ex vivo*, como parâmetro da extensão de estresse oxidativo e atividade antioxidante dos objetos de estudo.

2.3 Compostos naturais e sintéticos com propriedades antioxidantes

O termo antioxidante é amplamente utilizado e apresenta diferentes aplicações nas áreas da ciência. Na ciência e tecnologia de alimentos, um antioxidante é aplicado para inibir principalmente a peroxidação lipídica, reação que

compromete as características de qualidade dos alimentos devido a ocorrência de rancidez. Segundo Halliwell (2001), um antioxidante é qualquer substância que presente em baixa concentração em relação ao substrato oxidável, inibe ou previne significativamente a oxidação do substrato. O subtrato oxidável compreende todo tipo de molécula encontrada no sistema avaliado, a qual é alvo dos processos oxidativos.

Grande parte dos compostos naturais, com atribuições biológicas, são produtos do metabolismo secundário ou especializado das plantas. Os metabólitos especializados das plantas representam classes diversas de moléculas orgânicas, as quais não participam diretamento do metabolismo basal, que envolve seu crescimento e desenvolvimento como os metabólitos primários (carboidratos, proteínas, lipídios e ácidos nucléicos) (DEWICK, 2009) Ao contrários destes, os metabólitos secundários têm uma distruibuição limitada na natureza, são encontrados em um organismo específico ou em grupos, e desempenham algum papel vital às plantas, embora muitos sejam desconhecidos. (HARTMAN et al., 2007).

Nos próximos itens serão abordados aspectos químicos e biológicos de moléculas de ocorrência natural, sintéticas e semi-sintéticas assim como das espécies frutíferas nativas *P. cattleianum*, *Butia odorata* e *Campomanesia xanthocarpa.*

2.3.1 Deidrozingerona e compostos análogos

A deidrozingerona (4-hidroxi-3-metoxibenzalacetona, DHZ) (Figura 4) é uma molécula de ocorrência natural considerada um dos componentes bioativos e minoritários do gengibre, as raízes da *Zingiber officinalis* Roscoe. Os compostos que conferem a característica sensorial de pungência do gengibre são a zingerona, gingeróis, gingerdióis, gingerdionas e shogaóis (Figura 4) (SHIH et al., 2014).

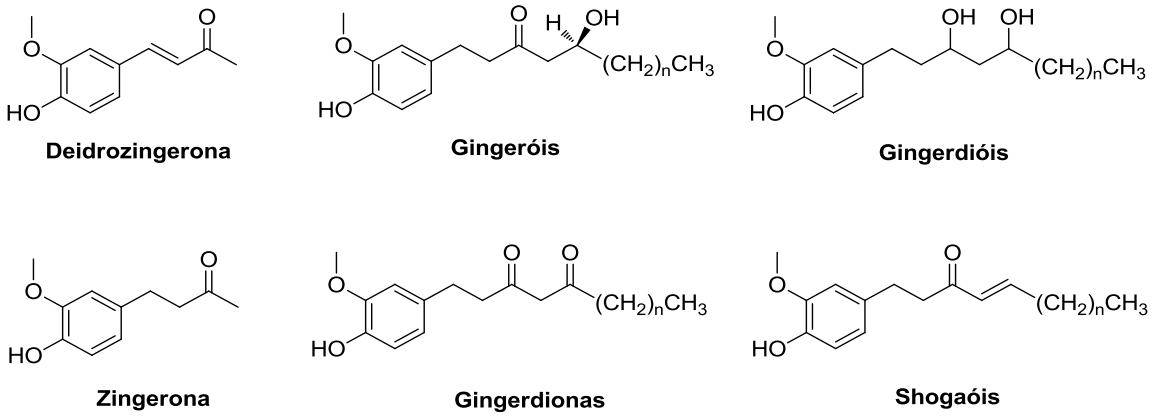


Figura 4 – Estruturas dos compostos identificados no gengibre, *Zingiber officinalis* Roscoe. n = 6, 8, 10.Fonte: Shih et al. (2014).

A DHZ é também um composto análogo à curcumina, que é o principal composto bioativo das raízes de *Curcuma longa* e que confere a cor amarelada ao condimento conhecido como açafrão (WANG et al., 1997). A curcumina tem um largo espectro de atividades biológicas, dentre as quais se destaca a capacidade de neutralizar espécies reactivas (AGGARWAL e HARIKUMAR, 2009; CHEN et al., 2006; MARCHIANI et al., 2013). A curcumina possui baixa solubilidade em soluções aquosas e fisiológicas e degrada-se rapidamente à estruturas como o ácido ferúlico, vanilina e a DHZ. Assim como a DHZ, os demais metabólitos da curcumina são metóxifenóis substituídos nas posições 2 e 4 do anel aromático que incluem uma grande classe de compostos de ocorrência natural.

Recentemente, Kancheva et al. (2014) demonstraram que tanto a DHZ como o dímero da DHZ protegem os sistemas de auto-oxidação lipídica e atuam de forma sinérgica quando combinados com o *α*-tocoferol, o que pode estar relacionado com a capacidade de regeneração desde último.

As principais características estruturais da DHZ, que justificam sua atividade antioxidante, são a presença de um grupo OH fenólico, que é capaz de neutralizar espécies radicalares pela transferência de um átomo de H; a presença de um grupo *orto*-metóxi doador de elétrons, o qual facilita a abstração de um átomo de H do grupo OH; a presença da cadeia alifática com uma ligação dupla que é capaz de estabilizar o radical fenoxil pela deslocalização de elétrons, proveniente da primeira reação (liberação de um próton H^+ do grupo OH fenólico)(KANCHEVA et al., 2013).

A importância do grupo OH fenólico da DHZ sobre a atividade antioxidante foi reportada por Kubra et al. (2014), onde os autores comparam sua atividade à de estruturas sintéticas derivadas pela substituição deste grupo por glicopiranosídeos, metila ou acetila. Os autores também sugerem que a presença do grupo carbonila *α,β*-insaturado em todas as moléculas é importante para as atividades antimicrobiana e antifúngica desses compostos. Esta hipótese também foi aventada pelo mesmo grupo (KUBRA et al., 2012), quando apresentaram pela primeira vez a capacidade antifúngica da DHZ.

A atividade antioxidante da DHZ é relatada na literatura em diversos modelos experimentais, como na auto-oxidação do ácido linoléico (LITWINIENKO et al., 2007), na proteção das membranas de células sanguíneas, frente a radicais livres sintéticos DPPH (YAMAGAMI et al., 2004; LITWINIENKO e INGOLD, 2004), radicais ABTS, e potencial redutor do íon férrico (Fe^{3+}) (PARIHAR et al., 2007).

Neste sentido, a DHZ e outros compostos fenólicos naturais, como o eugenol e o isoeugenol, apresentam potencial antioxidante em sistemas de peroxidação lipídica induzida em cérebro de ratos e frente a espécies oxidantes geradas por radiólise pulsada (hidroxil, radicais peroxil halogenados e radicais azida) (PRIYADARSINI et al., 1998). Priyadarsini et al. (1999) demonstraram que as formas proteolíticas da DHZ são dependentes do pH, predominando a estrutura fenólica e o íon fenóxido ($DHZO^-$) em pH < 8. Os autores sugerem que o íon fenóxido é mais eficiente que a estrutura fenólica da DHZ em doar um elétron a espécies oxidantes, como radicais peroxil e $NO_2^{\bullet}$ e que o radical fenoxil gerado é estável e, portanto, não apresenta capacidade pró-oxidante.

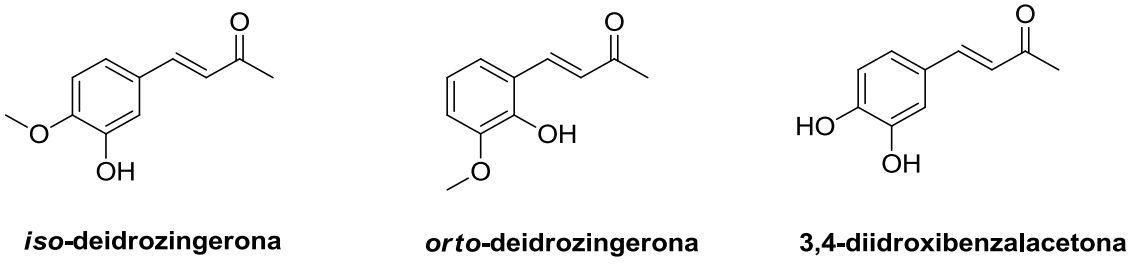
Diferentes compostos da classe das hidroxibenzalacetonas, incluindo a DHZ, têm a capacidade de inibir a oxidação lipídica da membrana de células sanguíneas e do DNA de células bacterianas. Estes mecanismos foram demonstrados por Motohashi et al. (2005), onde a atvidade antioxidante relacionou-se diretamente com a presença de substituintes doadores de elétrons na ordem OH > OCH_3 > CH_3, localizados na posição *orto* à OH fenólica.

Em relação ao potencial biológico *in vivo*, a DHZ apresentou atividade antioxidante, radioprotetora e antimutagênica quando administrada por via oral em camundongos submetidos à irradiação gama (PARIHAR et al., 2007). O efeito do

pré-tratamento com a DHZ também foi capaz de aumentar as defesas antioxidantes endógenas como GSH, GST e SOD nos animais. A administração de diferentes doses de DHZ por via oral ou tópica também apresentou capacidade cicatrizante e antioxidante em ratos (RAO et al., 2011a).

Outras propriedades biológicas já foram atribuídas à DHZ, como a atividade antifúngica e citotóxica (KUBRA et al., 2012; YOGOSAWA et al., 2012). Esta última foi demonstrada pela atividade antiproliferativa da DHZ e seus isômeros *iso*-DHZ e *orto*-DHZ (Figura 5) em células HT-29 de carcinoma de cólon. O mecanismo sugerido pelos autores envolve um acúmulo de ERO causado pela DHZ (250 e 500 μM), interrupção do ciclo celular e indução de apoptose (YOGOSAWA et al., 2012).

Uma das moléculas de ocorrência natural que possuem estrutura análoga à DHZ é a 3,4-diidroxibenzalacetona (Figura 5). Esta molécula é isolada a partir de extratos orgânicos do fungo Chaga. Seus extratos têm aplicação na medicina tradicional na Rússia para o tratamento de desordens gástricas e câncer SHASHKINA et al., 2006). A 3,4-diidroxibenzalacetona é considerada o principal componente antioxidante do Chaga (NAKAJIMA et al., 2007, 2009), além de ter propriedades anti-inflamatória (SUNG et al., 2008), anti-tumorigênica (NAKAJIMA et al., 2009) e neuroprotetora (GUNGIMA et al., 2014).



***iso*-deidrozingerona** ***orto*-deidrozingerona** **3,4-diidroxibenzalacetona**

Figura 5 - Estrutura dos isômeros *iso*-/*orto*-deidrozingerona e da 3,4-diidroxibenzalacetona.

A zingerona (Figura 4), assim como a DHZ, é relatada na literatura como um dos compostos bioativos do gengibre. Isto deve-se as evidências de suas propriedades antioxidante e anti-inflamatória em ratos (KIM et al., 2010). A zingerona é capaz de induzir a atvidade da SOD *in vivo* (KABUTO et al., 2005) de neutralizar $ONOO^-$ *in vitro* (SHIN, 2005) e de proteger contra danos induzidos por radiação *in*

vivo (RAO et al., 2009) e *in vitro* (RAO et al., 2011b) e inibir o estresse oxidativo e doenças associadas à síndrome do intestino irritável (BANJI et al., 2014).

2.3.2 Compostos orgânicos de selênio e enxofre

O selênio (Se) foi reconhecido como um micronutriente essencial para os mamíferos em 1950 (SCHWARZ, 1978) e em 1973 foi identificada a primeira selenoproteína, a GPx (FLOHÉ et al., 1973; ROTRUCK et al., 1973). Até o presente, 25 enzimas são classificadas como selenoenzimas, as quais contêm resíduos de selenocisteína no sítio ativo. As selenoenzimas desempenham funções fisiológicas importantes como antioxidante (GPx e tiorredoxina redutases), transporte de Se (selenoproteína P) e produção do hormônio tireóide (iodotironina deiodinases).

O Se é essencial na dieta humana. Em áreas de deficiência endêmica deste elemento, há a incidência da doença de Keshan, uma cardiomiopatia, e da doença de Kashin-Beck, uma osteoatropatia (GE et al., 1985; MORENO-REYES et al.,1998). Além disso, baixos níveis de Se no organismo podem ocasionar convulsões febris (MAHYAR et al., 2010), déficit cognitivo e relacionar-se com doenças neurodegenerativas, como Alzheimer (CORRIGAN et al., 1991).

Por outro lado, a ingestão excessiva de compostos orgânicos/inorgânicos de Se, causa sintomas como queda de cabelo e unhas, lesões na pele e no sistema nervoso, fadiga e irritabilidade. Enquanto que uma toxicidade aguda, causa lesões neuronais severas, gastrointestinal, respiratória, desordens cardiovasculares, entre outros (NAVARRO-ALARCON e CABRERA-VIQUE, 2008).

A ingestão média de Se recomendada para adultos é de 53 µg/dia para mulheres e de 60 µg/dia para homens (RAYMAN, 2012). Em países como Austrália e Nova Zelândia, a recomendação é de 60 µg/dia para mulheres, e de 70 µg/dia para homens, com limite máximo que pode ser ingerido de 400 µg/dia (AGDHE, 2005).

Vários autores revisaram nos últimos anos diferentes aspectos das espécies contendo Se e sua atividade biológica, incluindo a farmacologia de compostos orgânicos sintéticos contendo Se (SORIANO-GARCIA, 2004; NOGUEIRA e ROCHA, 2011); as atividades antioxidante e pró-oxidante de espécies inorgânicas de Se e de compostos oxo-selênio; espécies de Se em alimentos e suplementos; assim como

seu metabolismo em organismos mamíferos (RAYMAN, 2012; GAMMELGAARD et al., 2011).

Em alimentos, o Se é encontrado tanto nas formas inorgânicas quanto orgânicas como por exemplo, selenito, selenometionina, metilselenocisteína e o disseleneto de cisteína (KUMAR e PRIYADARSINI, 2014).

As formas orgânicas de Se selenometionina e metilselenometionina são encontradas principalmente em alimentos como grãos e vegetais. As famílias *Allium* e *Crussiferae*, que incluem o alho, a cebola e o brócolis, são fonte de metilselenocisteína, enquanto que o arroz é considerado uma importante fonte de Se na dieta, pela presença de selenometionina (~80%) e pequenas concentrações de metilselenocisteína, selenocisteína e selenito (WILLIAMS et al., 2009; SUN et al., 2010). A castanha do Brasil é considerada a melhor fonte de Se da dieta, pois apresenta aproximadamente 36 µg/g enquanto que em outros alimentos os níveis variam entre 0,01 - 1 µg/g (YANG, 2009).

Os compostos de Se encontrados na dieta podem ser divididos em ativos redox (selenetos e disseleneto de cisteína) e não redox (selenometionina e metilselenocisteína), o que ajuda a entender suas propriedades antioxidantes. Os compostos de Se como selenolatos ($R\text{-}Se^-$ e HSe^-) e disselenetos (R-Se-Se-R) são ativos redox. Todas as espécies de Se encontradas em alimentos podem potencialmente gerar selenolatos (HSe^-) e metil-selenolatos ($MeSe^-$), que possuem importante papel no metabolismo oxidativo, assim como os tióis e tiorredoxinas. Os selenolatos são espécies altamente nucleofílicas, enquanto que os selenóxidos são eletrofílicos. Esta combinação de reatividade é responsável pela atividade GPx-*like* de compostos orgânicos de Se (WEEKLEY e HARRIS, 2013).

Além disso, algumas características do elemento Se são relevantes neste estudo. Quando comparado ao enxofre (S), o Se possui maior raio atômico, o que resulta em ligações com carbono e hidrogênio mais fracas do que em compostos análogos contendo S.

A maior parte dos mecanismos pelos quais o Se desempenha as propriedades biológicas são através da atividades antioxidante e pró-oxidante. Neste sentido, a atividade antioxidante de compostos de Se deve-se à expressão de selenoproteínas antioxidantes, à atividade GPx-*like*, à neutralização de radicais livres e à ligação com metais. Estes mecanismos são geralmente associados aos

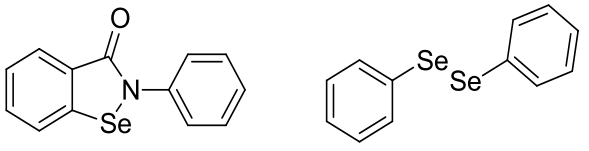
efeitos da suplementação de Se na prevenção de doenças, devido à relação entre estresse oxidativo e diversas patologias (WEEKLEY e HARRIS, 2013). Muitos compostos de Se de baixo peso molecular têm a capacidade de neutralizar radicais livres, a qual é atribuída à nucleofilicidade de selenolatos (RSe^-/HSe^-). Os selenóxidos, ácido selênico (RSeOH) e selenínico ($RSeO_2H$), gerados pela oxidação de selenolatos, são facilmente reduzidos por tióis celulares e dependentes de sistemas NADPH redutase (RAHMANTO e DAVIES, 2012; DE SILVA et. al, 2004).

A atividade pró-oxidante dos compostos de Se, por sua vez, está associada à oxidação do grupo tiol (SH) de proteínas e à geração de ERO, sendo que estas são as responsáveis pela citotoxicidade e atividade anticâncer de alguns destes compostos.

Levando-se em consideração que moléculas que contêm Se podem atuar como nucleófilos e portanto como antioxidantes, o desenvolvimento de métodos sintéticos para a obtenção de compostos orgânicos de Se destaca-se em alguns estudos (ARTEEL e SIES, 2001).

Um dos compostos que se destacam em relação à atividade antioxidante é o Ebselen (Figura 5), uma selenil amida, que possui grande parte das suas funções biológicas atribuídas à sua atividade GPx-*like*, além de ter atividade anti-inflamatória e anti-câncer (ORIAN e TOPPO, 2014). O Ebselen tem sido usado como composto de referência pela baixa toxicidade em testes em humanos (DAWSON et al., 1995; YAMAGUCHI et al., 1998; SAITO et al., 1998; OGAWA et al., 1999).

Outra molécula que apresentou atividade antioxidante em diversos estudos é o disseleneto de difenila (Figura 6). Este composto tem a capacidade de proteger células sanguíneas humanas e o tecido cerebral de ratos dos danos oxidativos causados por um pró-oxidante, o nitroprussiato de sódio (POSSER et al., 2006). Além disso, o disseleneto de difenila apresenta atividade antioxidante contra a peroxidação lipídica em outros tecidos (ROSSATO, et al. 2002; MEOTTI et al., 2004).



ebselen **disseleneto de difenila**

Figura 6 – Estrutura do ebselen e disseleneto de difenila.

A classe de disselenetos de diarila apresenta significativa atividade antioxidante frente à peroxidação lipídica *in vitro*, como por exemplo em tecidos hepático de ratos, como foi demonstrado por Andersson et al. (1994). Os autores sugerem alguns mecanismos para a ação antioxidante desta classe, os quais incluem a sua reação com peróxidos lipídicos (1, 2) e a degradação nucleofílica de hidroperóxidos (3), gerando intermediários como radical cátion selenil ($ArSe^{\bullet+}$) e selenóxidos ($Ar_2Se=O$), como demonstrado na Figura 7.

$$Ar_2Se + LOO^{\bullet} \longrightarrow Ar_2Se^{\bullet+} + LOO^{-} \quad (1)$$

$$Ar_2Se + 2LOO^{\bullet} + H_2O \longrightarrow Ar_2Se=O + 2LOOH \quad (2)$$

$$Ar_2Se + LOOH \longrightarrow Ar_2Se=O + LOH \quad (3)$$

Figura 7 – Mecanismos de ação antioxidante de diaril selenetos. Ar: C_6H_5; $LOO^{\bullet}$: radical peróxil de um ácido graxo insaturado; LOO^{-}: ánion peróxido; LOOH: hidroperóxido; LOH: álcool correspondente. Fonte: adaptado de Andersson et al. (1994).

De acordo com Nogueira et al. (2010), as propriedades antioxidantes exibidas pelos disselenetos de diarila estão associadas com a formação de intermediários benzenosselenol, que são nucleófilos, após a reação com tióis. Porém, o selenol formado ainda é pouco estável quando comparado aos originados pelos resíduos de selenocisteína da GPx.

A toxicologia e farmacologia de compostos orgânicos de Se foi extensamente revisada por Nogueira e Rocha (2011). Os autores relatam que os compostos inorgânicos de Se apresentam toxicidade relacionada à interação com grupos tióis (-SH) endógenos, quando comparados aos orgânicos.

O enxofre (S), assim como o Se, é um elemento não-metal presente no grupo 16 (ou 6a) da tabela periódica dos elementos químicos. O S é o principal elemento inorgânico essencial para todos os organismos biológicos. Isto deve-se à sua incorporação nas estruturas de aminoácidos, proteínas, enzimas, vitaminas e outras biomoléculas. As plantas, ao contrário dos seres humanos, podem utilizar S inorgânico e sintetizar aminoácidos como metionina e cisteína. Portanto, as plantas são fontes dietéticas de S (BAKER, 1977). O S faz parte da composição de diversos compostos com atividade biológica (Figura 8) como vitaminas [tiamina (vitamina B1) e biotina], aminoácidos (metionina, cisteína, cistina, taurina), proteínas, coenzimas (coenzima A, ácido lipóico), agentes redutores (glutationa reduzida), conservantes de alimentos (dióxido de enxofre, SO_2) e fertilizantes (sulfato de amônio).

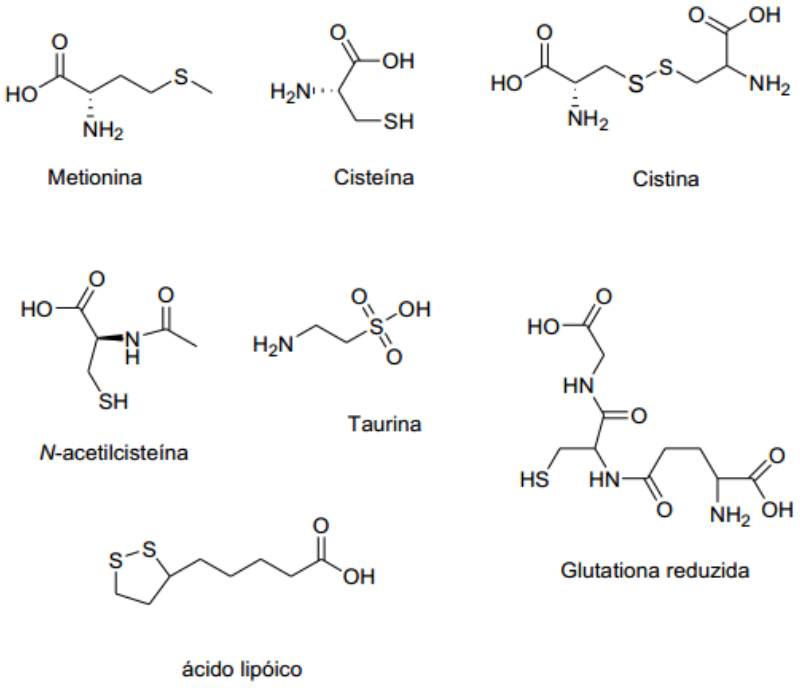


Figura 8 - Compostos orgânicos de enxofre biologicamente ativos.

Não há recomendação de consumo diário de enxofre, assim como a deficiência nutricional não é comum. Embora isto ocorra, a toxicidade do S é relativamente alta, em sua maior parte devido à sensibilidade ao sulfito (asma e choque anafilático) e ao SO_2. A ingestão de antioxidantes como as vitaminas C e E, carotenos e GSH pode ser necessária para aumentar a imunidade em pessoas que possuem intoxicação por S e para prevenir efeitos tóxicos (MEYDANI et al., 1995).

Dentre as diversas formas de ocorrência e bioatividade do S, no contexto do estudo da atividade antioxidante, a mais relevante é sua interconversão entre as formas dissulfeto e grupos sulfidril nas reações de oxidação-redução da glutationa no ciclo catalítico da GPx e da GST (Figura 9) (KOMARNISKY et al., 2003).

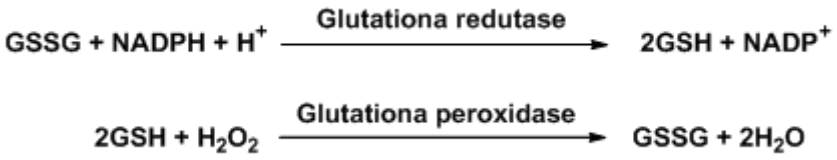


Figure 9 – Interconversão das formas dissulfeto (S-S) e sulfidril (SH) da GSH em ciclos catalíticos da GST e GPx. Fonte: Komarnisky et al. (2003).

Além da GSH, outras moléculas de ocorrência natural como a cisteína, ácido lipóico; e sintéticas como a *N*-acetilcisteína, têm efeito protetor contra o estresse oxidativo em sistemas biológicos devido à capacidade de neutralizar e reduzir vários agentes oxidantes (EISERICH e SHIBAMOTO, 1993).

Alguns compostos orgânicos de S encontrados no alho possuem importante função antioxidante, como exemplo: sulfeto e dissulfeto de dialila, *S*-alilcisteína, *S*-etilcisteína e *S*-propilcisteína. Estas moléculas têm a capacidade de proteger o plasma e as lipoproteínas de baixa densidade (LDL) de reações oxidativas e de glicação. Mais especificamente, isto estas moléculas também apresentam a capacidade de proteger contra a diminuição da atividade da CAT e GPx e aumentar da retenção de *α*-tocoferol no LDL em humanos (HUANG et al., 2004).

Além dos naturais, os compostos sintéticos de S também apresentam potencial biológico relacionado à sua atividade antioxidante. Recentemente Ianiski et al (2014) demonstrou a capacidade antioxidante *ex vivo* de um divinil sulfeto assimétrico [(4-*tert*-butilciclohexilideno)metil)(4-metoxistiril) sulfeto] contra o estresse oxidativo induzido no cérebro de camundongos.

Com base nas atividades biológicas apresentadas por compostos contendo S, este trabalho objetivou a síntese de novas moléculas a paritr da adição de grupos orgânicos contendo calcogênios à estruturas de moléculas de ocorrência natural. Neste sentido,exemplos que contemplam esta estratégia estão apresentados no item 2.4.

2.4 Compostos semi-sintéticos contendo calcogênios

A essencialidade e atividade biológica dos elementos Se e S são um dos fatores de maior importância no estudo de compostos organocalcogênios, como exposto no item 2.3.2. Diante da busca de novas moléculas com potencial antioxidante, antimicrobiano e neuroprotetor, compostos de ocorrência natural como aqueles encontrados em óleos essenciais apresentam expressivos estudos neste sentido (ADORJAN et al., 2010; DOBETSBERGER e BUCHBAUER, 2011). Dentre estes, destacam-se terpenos, sesquiterpenos, álcoois e aldeídos (BAKKALI et al., 2008).

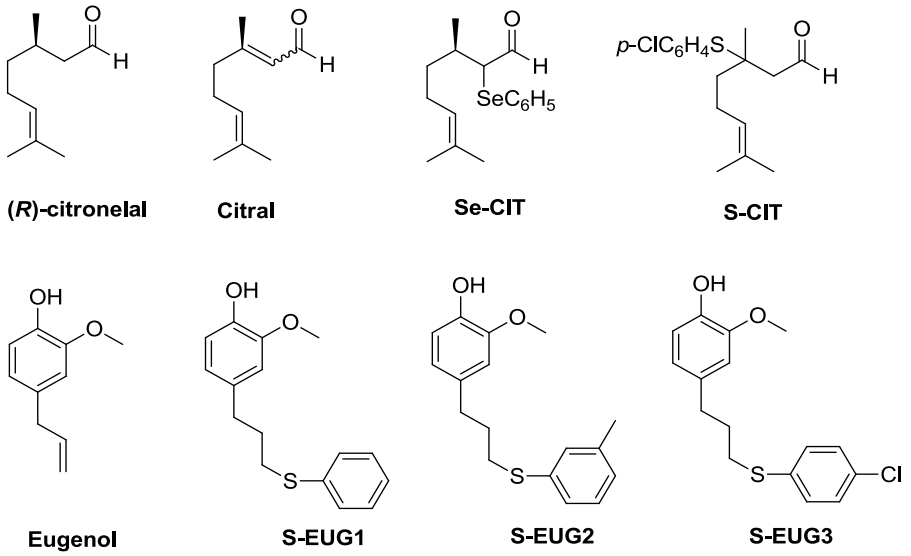


Figura 10 – Estrutura química do (*R*)-citronelal, Citral, Eugenol e compostos organocalcogênios análogos.

Estratégias sintéticas vêm sendo desenvolvidas a fim de gerar novos compostos a partir da combinação de dois ou mais compostos bioativos de ocorrência natural e/ou sintética. Esta estratégia, muitas vezes é efetiva para a obtenção de maior potencial ou atribuição de funções biológicas às novas estruturas (SHI et al., 2001; TATSUZAKI et al., 2007; NAKAGAWA-GOTO et al., 2007). Neste sentido, nosso grupo de pesquisa vem trabalhando com a modificação estrutural de compostos majoritários de óleos essenciais funcionalizados com grupos orgânicos contendo Se e S. Como exemplo, a modificação estrutural do citronelal, o principal

componente do óleo essencial de citronela (*Cymbopogon nardus*), a partir da adição de um grupo Se-fenil promoveu a obtenção do composto *α*-fenilseleno citronelal (**Se-CIT**) que possui atividade antibacteriana (Figura 10). A síntese do composto análogo *α*-fenilseleno citronelol foi também uma alternativa para a obtenção de um composto semi-sintético com atividade antibacteriana (VICTORIA et al., 2009; 2012).

Como exemplo de compostos orgânicos semi-sintéticos contendo S, nosso grupo descreveu recentemente alguns resultados promissores envolvendo a síntese de derivados do eugenol. O eugenol é um constituinte majoritário do óleo essencial de cravo-da-india (*Eugenia caryophillata*). Os compostos derivados desta estrutura (**S-EUG1-3**) exibiram potencial antioxidante *in vitro* superior à do eugenol e do antioxidante sintético BHA em um sistema de peroxidação lipídica (LENARDÃO et al., 2013). Outro exemplo, foi a inserção de um grupo *p*-Cl-tiofenil ao citral, com a obtenção do composto 3-(*p*-clorofenil)tio citronelal (**S-CIT**) (Figura 10). Este composto apresentou atividade antibacteriana significativamente superior à do composto de partida (GOLDBECK et al., 2014).

Com base nessas considerações, este trabalho objetivou a síntese de compostos organocalcogênios semi-sintéticos a partir da modificação estrutural da DHZ e análogos. Para isso estratégias sintéticas foram utilizadas para a inserção de grupos Se-aril e S-aril e posterior avaliação das propriedades antioxidantes *in vitro* dos compostos sintetizados (Capítulo 4).

2.5 Espécies nativas frutíferas

Segundo a Organização das Nações Unidas para Alimentação e Agricultura (FAO), a produção mundial das principais frutas tropicais ocorre em países em desenvolvimento (98%), com pequena participação de países desenvolvidos (2%) (FAO, 2010). Nesse contexto, o Brasil é o terceiro maior produtor mundial de frutas, atrás da China e da Índia (FAO, 2010; MAPA, 2010). Embora o Brasil possua uma abundância natural de frutas tropicais, poucas espécies estão disponíveis no mercado, o que pode estar relacionado à falta de investimento em sistemas de produção e de conservação pós-colheita de frutas nativas (LETERME et al., 2006). Entretanto, uma grande variedade de frutas nativas e/ou exóticas é consumida no

país ainda que haja poucos estudos sobre a viabilidade de sua introdução nos mercados (MANICA, 2000).

As espécies nativas representam um amplo campo exploratório nas áreas da química, bioquímica e tecnológica, visando valorizar e melhorar o uso desses recursos naturais.

2.5.1 *Psidium cattleianum*

A família *Myrtaceae* é uma das mais importantes no Brasil em regiões tropicais e subtropicais. Representada por 121 gêneros e 3800-5800 espécies, contém plantas aromáticas frutíferas de importância na preservação da fauna local (STEFANELLO et al., 2011).

Dentre as espécies frutíferas nativas do Brasil desta família, a *Psidium cattleianum,* originária da América do Sul e Central, é conhecida pelos frutos denominados araçá (LORENZI, 1992). Os frutos têm safra nos meses de fevereiro a abril. O araçá possui características sensoriais intensas, como aroma e acidez e as bagas possuem coloração amarelada ou avermelhada, dependendo do genótipo (MANICA, 2000; LUXIMON-RAMMA, 2003).

A composição de fitoquímicos do araçá vermelho e amarelo foi explorada por Feter et al. (2010) evidenciando-se que a cultivar vermelha tem maiores níveis de antocianinas e carotenóides que a amarela. No mesmo estudo, os níveis de compostos fenólicos tiveram uma correlação positiva com a capacidade antioxidante *in vitro* do extrato metanólico dos frutos; porém, os níveis de antocianinas e carotenóides não exibiram a mesma correlação.

Luximon-Ramma et al. (2003) relataram elevados níveis de compostos fenólicos, flavonóides e proantocianidinas no araçá, que correlacionaram-se com a capacidade antioxidante *in vitro* dos frutos.

A capacidade antioxidante de extratos orgânicos (acetona) e aquosos de diferentes acessos de araçá também foram demonstrados por Medina et al. (2011). O estudo demonstrou que extratos orgânicos de genótipos de araçá vermelho mostraram capacidade antioxidante superior à do araçá amarelo frente a radicais livres sintéticos DPPH. Os principais compostos fenólicos identificados com potencial antioxidante em ambas cultivares foram (-)-epicatequina e ácido gálico, e

minoritários, os ácidos cumárico e ferúlico, a miricetina e a quercetina (Figura 11) (MEDINA et al., 2011).

No mesmo estudo, verificou-se que os extratos apresentaram capacidade de diminuir a citotoxicidade induzida por peróxido de hidrogênio (H_2O_2) em cepa de *Sacharomyces cerevisiae* e de diminuir o crescimento de células tumorais de adenocarcinoma de mama (MCF-7). Também foi demonstrada a atividade antimicrobiana de extratos de araçá frente ao crescimento de *Streptococcus mutans* e *Salmonella enteritidis* (MENEZES et al, 2010; MEDINA et al. 2011).

Recentemente, um estudo realizado por McCook-Russel et al. (2012) elucidou o potencial anti-inflamatório *in vitro* de extratos orgânicos de araçá. A partir da obtenção de extratos com hexano e acetato de etila dos frutos de araçá, os autores isolaram como fitoquímicos bioativos os ácidos ursólico e oleanólico (Figura 11), os quais possuem atividade anti-inflamatória em modelos experimentais *in vivo* (LIU, 1995). Os extratos exibiram a capacidade de inibir seletivamente a cicloxigenase tipo 2 (COX-2), que catalisa a reação de oxidação do ácido araquidônico a prostaglandinas e tromboxanos nos processos inflamatórios (BOWEN-FORBES et al., 2009).

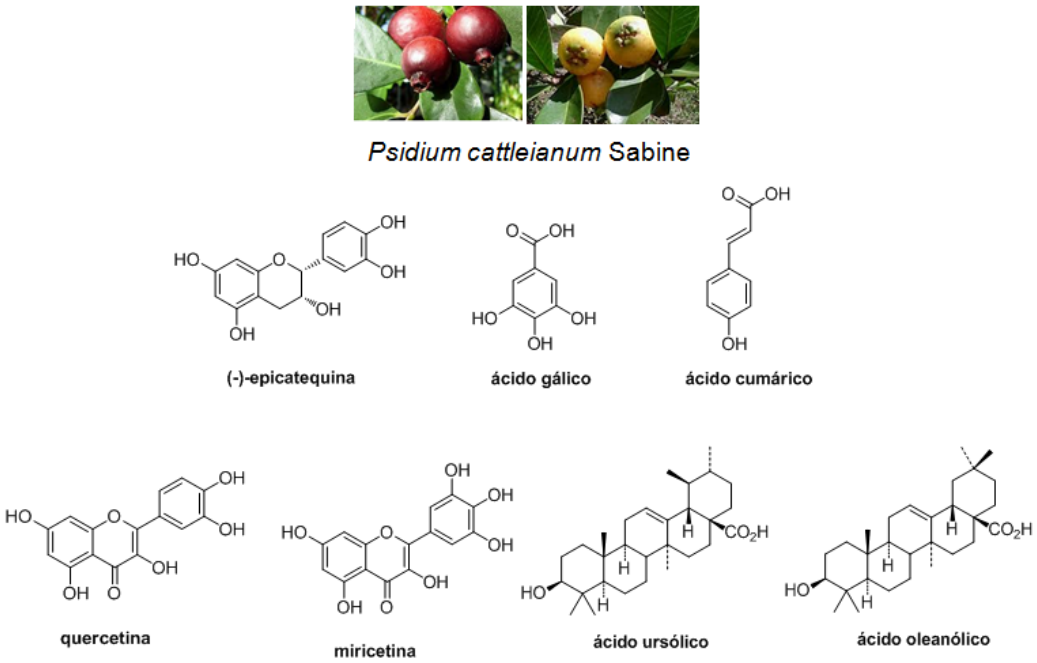


Figura 11 – Principais fitoquímicos identificados em frutos de *Psidium cattleianum* Sabine - araçá.

Em relação à toxicidade, existem apenas estudos referentes aos extratos das folhas desta espécie. O extrato hidro-alcoólico, por exemplo, não apresentou mutagenicidade e citotoxicidade sobre o DNA de leucócitos e células da medula óssea de ratos, segundo um estudo realizado por Costa et al. (2008).

Por outro lado, significativa citotoxicidade foi observada em linhagem de carcinoma gástrico tratada com extrato das folhas de araçá constituído de fitoquímicos como ácido ferúlico, genisteína e 3,4,5-trimetoxiflavona (MOON et al. 2011). A partir do extrato metanólico das folhas de *P. cattleianum*, verificou-se a capacidade antimicrobiana contra *Sthaphylococcus epidermidis, Bacilus subtilis* e *Micrococcus luteus*, como demonstrado por Souza et al. (2004).

Com base nos dados presentes na literatura até o momento, evidencia-se o potencial biológico dos componentes fitoquímicos dos frutos do araçá e verificou-se a ausência de estudos relacionados a propriedades antioxidantes *in vitro* mais específicas e também de dados sobre os efeitos *in vivo*, incluindo parâmetros de toxicidade e estresse oxidativo a partir consumo dessa fruta.

2.5.2 *Campomanesia xanthocarpa*

A espécie *Campomanesia xanthocarpa* O. Berg, também da família *Myrtaceae*, é popularmente conhecida por seus frutos denominados guabiroba e encontra-se como planta nativa na região sul do Brasil, na Argentina, no Paraguai e no Uruguai (LORENZI, 1992). A frutificação da guabirobeira se dá de dezembro a maio e seus frutos são ricos em vitaminas e compostos aromáticos. Contudo, são escassos os estudos sobre a atividade biológica da *C. xanthocarpa* (VALLILO et al., 2006).

A infusão das folhas desta planta é utilizada popularmente para o tratamento de doenças inflamatórias, para a perda de peso e hipercolesterolemia (ALICE et al., 1995; DICKEL et al., 2007). Evidências científicas apontam alguns efeitos farmacológicos de extratos de suas folhas, incluindo a redução do colesterol e LDL sanguíneos em pacientes hipercolesterolêmicos pelo consumo de cápsulas (KLAFKE et al., 2010); prevenção de úlceras gástricas induzidas por etanol em ratos pelo tratamento com extrato hidroalcoólico (MARKMAN et al, 2004); diminuição dos níveis de glicose sanguíneos em ratos diabéticos pelo tratamento com extrato

aquoso (VINAGRE et al. 2010) e recentemente foi demonstrada a capacidade antiplaquetária, antitrombótica e fibrinolítica do extrato aquoso em camundongos (KLAFKE et al., 2012).

O óleo essencial das folhas de *C. xanthocarpa* foi caracterizado por Limberger et al. (2001), os quais identificaram os compostos linalol e (*E*)-nerolidol como constituintes majoritários (Figura 12). Enquanto que o óleo essencial das flores apresentou o monoterpeno ledol como constituinte principal (CARDOSO et al., 2010). O óleo essencial dos frutos de *C. xanthocarpa* possui como constituintes principais os monoterpenos α-pineno, *o*-cimeno, *β*-pineno, limoneno e *β*-cariofileno (MARIN et al., 2008; VALLILO et al., 2008).

Os óleos essenciais de diferentes partes da *C. xanthocarpa* ainda não foram explorados quanto às suas propriedades biológicas e toxicológicas, tornando-se um produto natural de interesse em investigações científicas nesse sentido.

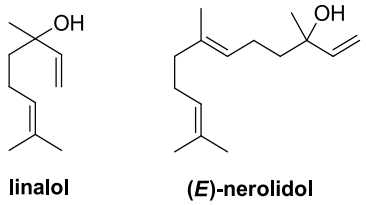


Figura 12 – Constituintes majoritários do óleo essencial das folhas de *C. xanthocarpa* O Berg, de acordo com Limberger et al. (2001).

A composição química dos frutos de *C. xanthocarpa* os classifica como uma fonte de ácido ascórbico e compostos fenólicos na dieta, podendo exibir efeitos nutracêuticos em função da sua alta capacidade antioxidante (PEREIRA et al., 2012). Os níveis de ácido ascórbico da guabiroba são muito superiores aos de outros frutos nativos, como araçá amarelo e a uvaia, como foi evidenciado por Pereira et al. (2012). No mesmo estudo, os extratos dos frutos se destacaram

quanto ao teor de carotenóides (zeaxantina, *β*-caroteno e criptoxantina) e à sua capacidade antioxidante.

Com base nesses dados, pode-se inferir que os extratos obtidos de diferentes partes da *C. xanthocarpa* possuem fitoquímicos de importância biológica. Neste sentido, o potencial biológico do óleo essencial das folhas, dentre os estudos citados acima até o momento, são escassos na literatura.

2.5.3 *Butia odorata*

O gênero Butia é pertencente à família *Palmae* ou *Aracaceae*, comumente chamadas de palmeiras. Este gênero compreende seis espécies presentes no sul do Brasil: *Butia odorata* Becc, *Butia capitata* Becc., *Butia eriospatha* Becc., *Butia paraguayensis*, *Butia yatay* Becc. e *Butia witeckii* (ROSSATO et al., 2007; SOARES e LONGHI, 2011). Seus frutos, denominados butiá, são disseminados na região sul do Brasil e possuem características sensoriais intensas como acidez, doçura, sabor e aroma frutais atraentes, permitem a preparação de diversos produtos alimentícios (Büttow, 2008). A grande diversidade genotípica dessas árvores, garante frutos de variados tamanhos e composição fitoquímica diversa (DAL MAGRO et al., 2006; ROSSATO et al., 2007). A coloração do mesocarpo dos butiás varia de amarelo claro a laranja avermelhado e fibroso. O endocarpo é duro e denso, além de conter de uma a três sementes oleaginosas. Os frutos têm período de maturação de dezembro a março (ROSA et al., 1998; PEDRON et al., 1998).

Considerando-se os alimentos naturalmente ricos em compostos funcionais, como antioxidantes, dentre as espécies botânicas nativas com potencial de exploração, a *B. odorata* apresenta algumas características interessantes. Neste sentido, os frutos apresentam significativos níveis de carotenóides e ácidos graxos essenciais (LOPES et al., 2011; FERRÃO et al., 2013). Ferrão et al. (2013) demonstraram o perfil de ácidos graxos de vários genótipos de frutos de *B. odorata* da região sul do Brasil, os quais compreendem majoritariamente os ácidos palmítico, linoléico e *α*-linoléico (Figura 12).

De acordo com o Instituto Americano de Medicina, 100 g da polpa de frutos da espécie *B. capitata* correspondem a 100% da necessidade diária de vitamina A (retinol) na dieta humana (IOM, 2001).

O perfil de carotenóides em frutos de *B. capitata* foi determinado por Faria et al. (2011), tendo predominantemente o *β*-caroteno como reprensentante desta classe. Os frutos de *B. capitata* também foram avaliados no estudo e se mostraram uma boa fonte de pró-vitamina A para a dieta humana. Pereira et al. (2013), quantificaram os níveis de ácido ascórbico e demonstraram o perfil de carotenóides presentes nos frutos de *B. capitata*, onde além do *β*-caroteno (50% do total de carotenóides), foram identificados seu isômero 9-cis-*β*-caroteno (25%) e também a luteína (11,8%) (Figura 13).

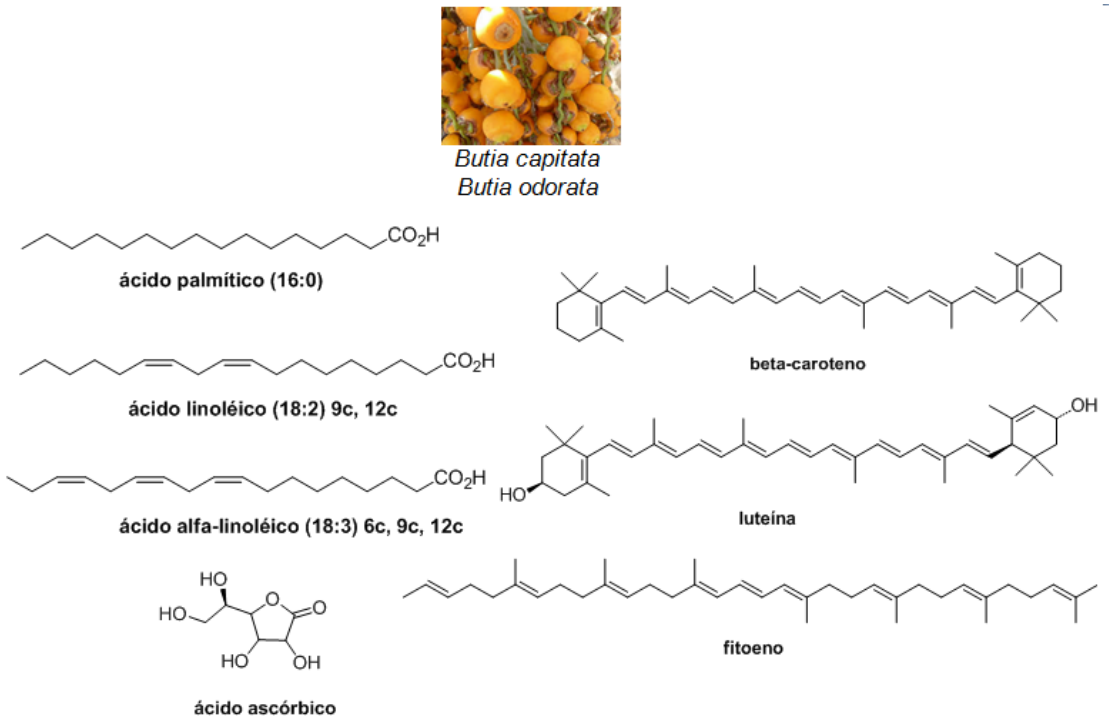


Figura 13 – Fitoquímicos identificados em frutos de *B. capitata* e *B. odorata.*

As propriedades antioxidantes dos frutos desta espécie também foram evidenciadas na literatura, em estudo realizado por Pereira et al. (2013), onde os butiás demonstraram maior potencial antioxidante *in vitro* frente a radicais livres que outros frutos nativos (PEREIRA et al., 2013).

Com base nos dados disponíveis na literatura com relação à sua composição química, ainda existe um amplo potencial biológico a ser investigado relacionado aos frutos da espécie *B. odorata*.

3 Estudo da síntese e atividade antioxidante *in vitro de* compostos organocalcogênios análogos à deidrozingerona

A partir da revisão da literatura sobre a DHZ e compostos organocalcogênio, este manuscrito descreve os resultados da síntese e atividade antioxidante *in vitro* de derivados da DHZ contendo selênio e enxofre. O objetivo foi a preparação de novas moléculas com potencial antioxidante derivadas da combinação de unidades estruturais com propriedades biológicas. Este trabalho foi desenvolvido em colaboração com o Prof. Carl Schiesser, supervisor do estágio de doutoral realizado na The University of Melbourne - Bio21 Institute. O manuscrito científico será submetido ao periódico *Organic & Biomolecular Chemistry*, ISSN: 1477-0520.

Synthesis and antioxidant properties of selenium and sulfur-containing zingerone analogues

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Abstract

Dehydrozingerone (4-hidroxy-3-methoxybenzalacetone) (DHZ) is a minor and bioactive constituent of ginger (*Zingiber officinalis* Roscoe). In this study a serie of DHZ derivatives was sinthesized including selenium and sulfur-containing compounds. The *in vitro* antioxidant activity of compounds was evaluated in several assays. Michael adducts containing Se and S-aryl groups were the most actives antioxidants. Some of DHZ derivatives compounds had the capactiy to delay the linoleic acid oxidation induxed by sodium nitroprusside and anti-hemolysis activity, which can be explained by their enhanced DPPH-scavenging. The chemical modification strategy was potentially valuable for the development of new antioxidants analogs to dehydrozingerone.
Keywords: dehydrozingerone, organoselenium, organosulfur, antioxidant, anti-hemolysis.

1. Introduction

Dehydrozingerone (DHZ, **1a**) and zingerone (**2a**) are minor phenolic compounds structurally related to curcumin (**3**) which are present in ginger, the rhizomes of *Zingiber officinale* Rosco.[1] DHZ, with vanillin and ferulic acid, also is formed as a degradation product of curcumin at physiological pH.[2] Ginger has widely been used both as ingredient and flavoring in foods, as well as a medicinal agent.[3] Several pharmacological studies have been reported for extracts[3b] or its constituents DHZ and zingerone.[4] Particularly, DHZ has radioprotective,[3a] antimicrobial,[4a,b] pro-healing,[4c] antioxidant,[2,4a,c-j] anti-Parkinson,[4d] antimutagenic,[4k] anti-cancer[4l,m] and antidiarrheal[4n] effects.

The unbalance of oxidative metabolism has a crucial role in the progression of chronic deseases. Reactive Oxygen Species (ROS) are constantly formed in mamalian systems[5] either as accidental products during physiologic processes,[5a] or due to environmental pollutants, such as ozone,[5b] heavy metal poisoning[5c] and ionizing radiation.[5d] If ROS are not controlled by cellular antioxidant defenses, they can generate a state of oxidative stress.[6] The resulting ROS are responsible for the progressive and irreversible decline

of various metabolic functions of the organism during aging, including fertility,[7a-c] dementia[7d] and cancer.[7e]

Besides being versatile intermediates in organic synthesis,[8] organochalcogen compounds frequently exhibit biological activity, including antioxidant, antinociceptive, anticancer, antidepressant, antibacterial, antifungal, among others.[9] Novel organochalcogen compounds were recently synthesized and proved to be strong antioxidants.[9,10] In this context, the antioxidant properties of organoselenium compounds, such as mono- and diselenides, have been demonstrated both in *in vitro* and *in vivo* models.[9]

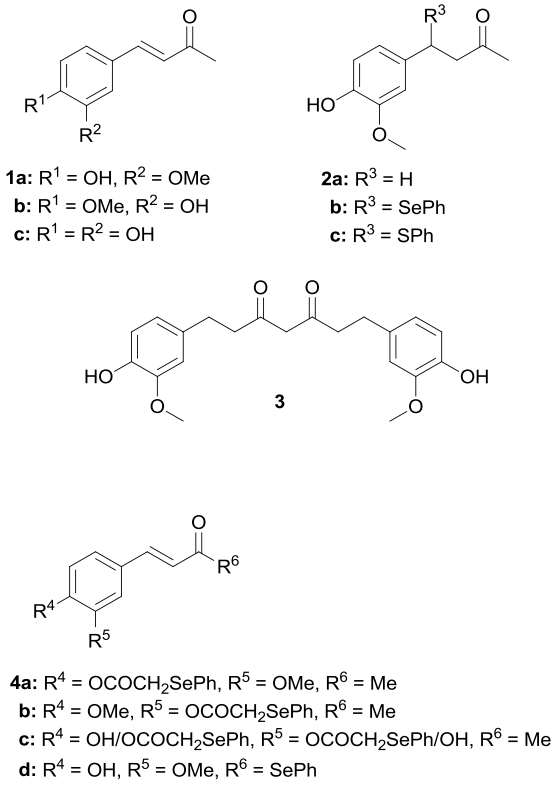


Fig. 1 – DHZ (**1a**), zingerone (**2a**) and analogues.

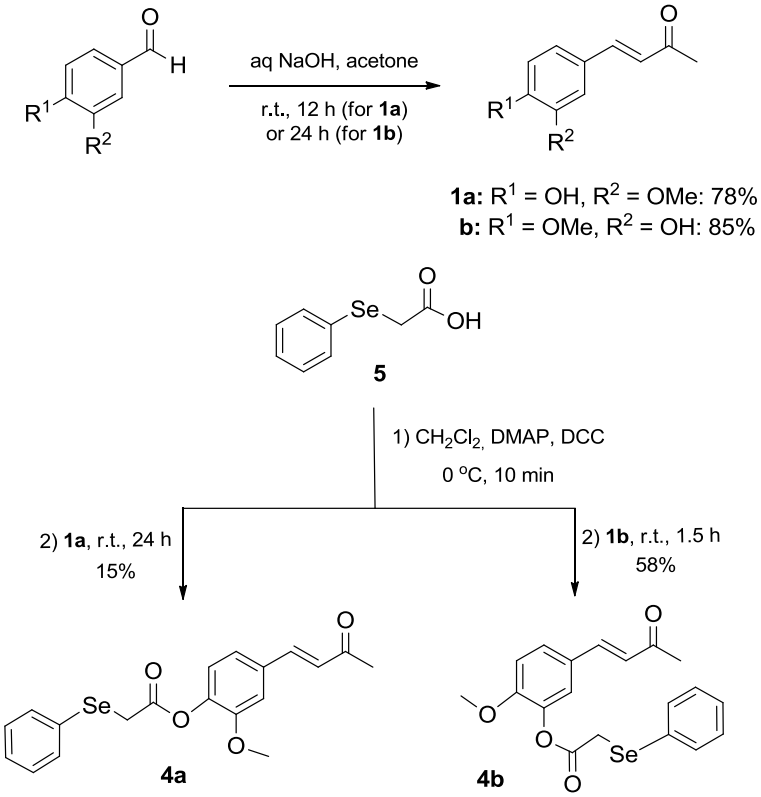
The combination of two or more bioactive compounds in one molecule has been used as an effective strategy for designing new drugs and promising results with different classes of compounds have been described.[11] Based on DHZ and chalcogen-containing compounds bioactivity, we have interest in the synthesis and biological activity of semi-synthetic organochalcogen compounds[12]. We present here our results on the synthesis and the antioxidant evaluation of new derivatives of DHZ and zingerone (**2**, **4**).

To that end, we developed synthetic strategies for the preparation of arylseleno-modified derivatives of DHZ (**1a**). Modified compounds included esters (**4a**-**c**), the ketone (**4d**), and compounds **2b**-**c**. The effect of these chemical modifications on the antioxidant capacity of the molecules in question was also examined. Methods employed include: the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$), nitric oxide (NO) and hydroxyl radicals ($^{\bullet}OH$) scavenging activities, ferric reducing antioxidant power (FRAP), linoleic acid peroxidation and anti-hemolysis activity assays. These assays provide important information about the hydrogen-transfer and electron-donating abilities of the compounds in question to free radicals, and their protective capacity against lipid peroxidation in homogeneous and heterogeneous environments.

2. Results and Discussion

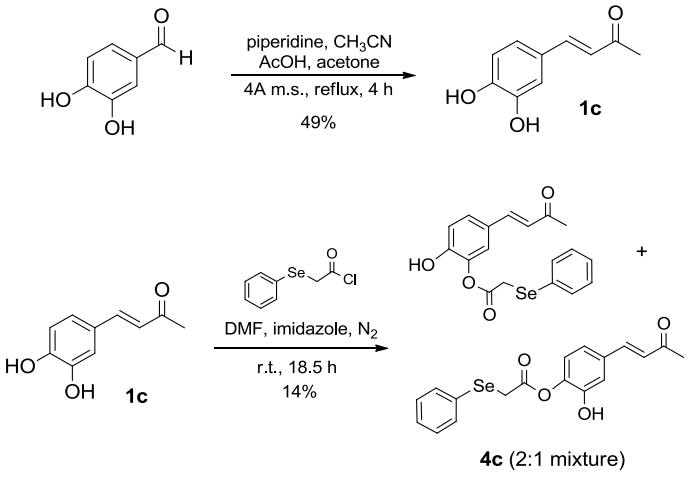
Synthesis of dehydrozingerone analogues

The overall strategy for the synthesis of the DHZ analogues (**2**, **4**) is outlined in Scheme 1-4. Compounds **1a**-**c** were prepared by aldol condensation of the appropriate benzaldehyde with acetone as described in the literature.[5i,13] Treatment of DHZ (**1a**) or *iso*-DHZ (**1b**) with 2-(phenylseleno)acetic acid (**5**)[14] in dichlormethane and in the presence of DCC afforded the esters (**4a**, **4b**) in low to moderate isolated yield (15 and 58% respectively), but in excellent yield based on recovered starting material (Scheme 1). This outcome reflects the difference in reactivity between the phenolic groups in **2a** and **2b**, with the lone pair in the former being more extensively delocalised and therefore less reactive.



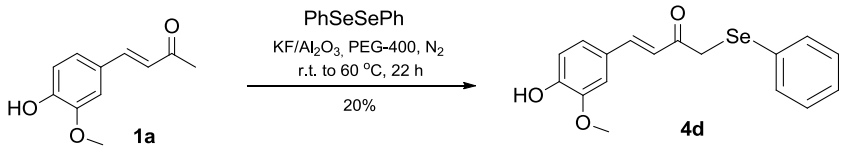
Scheme 1. Synthesis of DHZ (**1a**), *iso*-DHZ (**1b**), and *O*-acylated, Se-containing derivatives **4a** and **4b**.

Compound **4c** was isolated in low yield (14%) as an inseparable mixture of isomers (1:1) upon treatment of 3,4-dihydroxybenzalacetone (**2c**) with 2-(phenylseleno)acetyl chloride[15] in DMP and in the presence of imidazole (Scheme 2).



Scheme 2. Synthesis of 3,4-dihydroxybenzalacetone (**1c**) and Se-containing derivatives **4c**.

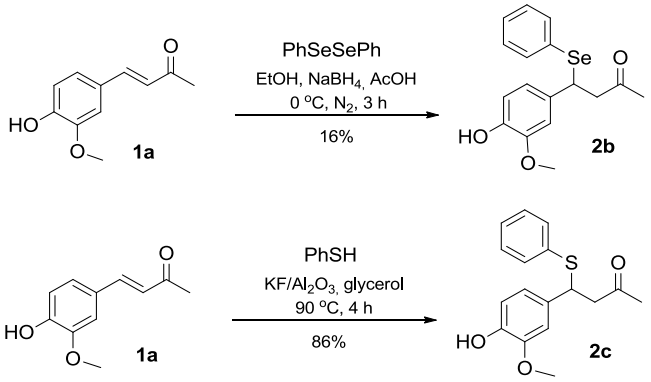
To prepare the *α*-phenylseleno-DHZ **4d**, solid supported KF wasmused as base and PEG-400 as the solvent to generate the enolate indermediate, which reacted with diphenyl diselenide (Scheme 3).[16] Despite the low yield of isolated product (20%), unreacted PhSeSePh and DHZ were easily recovered after the reaction.



Scheme 3. Synthesis of 1-phenylseleno-DHZ **4d**.

The conjugate addition of an organochalcogenium group to α,β-unsaturated carbonyl compounds is an efficient strategy for C–S and C–Se bond formation.[17] Traditional methods for thia-Michael addition requires activation of acceptor olefin by Lewis acid or deprotonation of thiol by base.[18] To prepare selenium-Michael adducts, the selenol is frequently generated *in situ* from diphenyl diselenide in acidic media,[19a] or by using the system $(PhSe)_2/NaBH_4$/PEG-400.[19b] Alternatively, the nucleophilic 1,4-addition of $C_6H_5SeZnCl$ on water was successfully employed toprepare β-phenylselenium ketones.[19c]

Thus, the treatment of dehydrozingerone (**1a**) with benzeneselenol afforded the Michael adduct 4-phenylseleno zingerone (**2b**) in 16% yield (Scheme 4). In this study, the best results were obtained when benzeneselenol was generated *in situ* from PhSeSePh and $NaBH_4$ in EtOH and AcOH.[19a] The acidic media was crucial to avoid the reduction of the carbonyl group of DHZ. Attempts to improve the yield of **2b** from **1a** using different methods to generate PhSeH failed.[19b,c,20] The fast decomposition of **2b** to zingerone (**2a**) and diphenyl diselenide or the competition for reduction of the carbonyl group contributed to the modest yield of the reaction. Due to its low stability, **2b** was purified by preparative TLC and was used immediately after purification in the antioxidant essays. Similarly, 4-phenylthio zingerone (**2c**) was prepared by the Michael addition of benzenethiol to DHZ in 86% yield, using solid-supported KF in the presence of glycerol as the solvent (Scheme 4).[18c]



Scheme 4. Synthesis of 3-phenylchalcogeno DHZ **2b** and **2c**.

Antioxidant activity of compounds evaluated by radicals scavenging activity and FRAP assay

A series of *in vitro* antioxidant screening methods were used to explore the possible antioxidant potential of synthesized compounds. The first assay was carried out to evaluate the interactions of molecules with DPPH radicals that reveal the ability of those to donate hydrogen atom to N-centered radicals. This process is not affected by metal ion chelation and enzyme inhibition.[21] The interaction with ABTS radicals reveals the ability of the antioxidant to reduce radicals by electron-tranfer reactions.[22] The reducing capacity by FRAP assay provides a measure of the ability of compounds to donate electrons and to reduce the transition metals as ferric ions. The electron donation capacity of some bioactive compounds is directly associated with their antioxidant activity.[23]

The IC_{50} (concentration for 50% radical scavenging) and stoichiometric factor *n* values as well as maximal inhibition (I_{max}) of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are represented in Table 1. According the IC_{50} and *n* values, the $DPPH^{\bullet}$ scavenging activity decreased in the order **4c**=**1c**>**2b**=**2c**>**1a**=**4d**>**1b**. On the other hand, compounds **4a** and **4b**, without phenolic OH group, had a very low DPPH scavenging activity (I_{max} 21-24%). Additionally, the position of OH group showed an important role to "DPPH trapping" as can be explained by difference of potential antioxidant between **1a** (IC_{50} = 57.0), **1b** (IC_{50} = 250.0) and **1c** (IC_{50} = 6.0 ± 1.5).

Similarly, the $ABTS^{\bullet+}$ scavenging activity of compounds (Table 1) decreased in the order **1c**=**4c**=**2c**>**2b**=**1a**>**1b**>**4d**, when IC_{50} and *n* values were compared. On the other hand, compounds **4a** and **4b** didn't scavenge $ABTS^{\bullet+}$.

Table 1 – DPPH and ABTS radicals scavenging activity of **1a**-**c**, **2b**-**c** and **4a**-**d**.

Compound	DPPH			ABTS		
	$I_{max\ (\%)}$	IC_{50} (μM)	*n*	$I_{max\ (\%)}$	IC_{50} (μM)	*n*
1a	82. 45 ± 3.6 [a]	57.0 ± 2.5 [a]	0.4 [a]	96.96 ± 1.9 [a]	8.0 ± 1.0 [a]	6.3 [a]
4a	24.73 ± 0.8 [b]	nd	nd	nd	nd	nd
1b	64.61 ± 4.2 [c]	250.0 ± 5.0 [b]	0.1 [b]	94.23 ± 5.5 [a]	11.5 ± 2.5 [b]	4.4 [b]
4b	21.07 ± 4.7 [b]	nd	nd	nd	nd	nd
1c	90.74 ± 9.2 [d]	6.0 ± 1.5 [c]	4.1 [c]	95.36 ± 3.4 [a]	6.0 ± 2.0 [c]	8.5 [c]
4c	93.13 ± 6.0 [d]	5.0 ± 1.0 [c]	5.0 [c]	89.19 ± 0.7 [b]	5.0 ± 2.0 [c]	10.2 [c]
4d	84.62 ± 2.3 [a]	62.0 ± 2.0 [a]	0.4 [a]	94.26 ± 5.0 [a]	24.0 ± 3.0 [d]	2.1 [d]
2b	96.61 ± 3.0 [e]	27.67 ± 9.6 [d]	0.9 [d]	99.51 ± 0.8 [c]	8 ± 1.0 [a]	6.3 [a]
2c	96.36 ± 3.3 [e]	33.3 ± 3.5 [d]	0.7 [d]	99.68 ± 0.5 [c]	6.5 ± 0.5 [c]	7.8 [c]

Data are expressed as mean ± SE of % maxinal inhibition (Imax) and noncentration to scavenge 50% of free radicals (IC_{50}) DPPH and $ABTS^{\cdot}$. *n* = stoichiometric factor (the equivalent of radicals scavenged by one equivalent of antioxidant). Different letters in the same column denote significant difference ($p < 0.05$) by Student-Newman-Keuls test for post-hoc comparison. (nd): not detected.

The reducing capacity of compounds was evaluated by the FRAP assay. The increase of absorbance denotes the reducing activity of the complex Fe^{3+}-triazine. Results are expressed as absorbance summarized in Table 2. Only the derivative **2c** showed a better reducing capacity than **1a**, while **4a** and **4b** without phenolic-OH group in their structures had not reducing ability, which is in accordance with the data from DPPH and ABTS radicals scavenging assays. As can be seen on Table 2, compounds **1c** and **2c** presented a FRAP higher than the limit of detection at 593 nm.

Several data indicated the antioxidant activity of **1a**, not only by scavenging DPPH and ABTS radicals,[24,25] but also against biologically important radicals, like hydroxyl ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$).[26] In this sense, the antioxidant of **1a** is atributed by hydrogen atom-transfer ability from OH-phenolic group to reactive species, and this facility is also attributed to an electron-withdrawing effect of its carbonyl group conjugated to the aromatic ring.[27] According Priyadarsini et al. (1999)[26], the anion phenoxide from **1a** is more efficient than the phenol group to inhibit the free radicals and phenoxyl radical is stable by aromatic ring ressonable effect.

Table 2 - Ferric reducing-antioxidant power (FRAP) assay of DHZ and derivatives.

Compound	Concentration (μM)				
	1	**10**	**50**	**100**	**500**
1a	0.145 ± 0.02	0.357 ± 0.23[a]	1.031 ± 0.48[c]	max	max
4a	nd	Nd	nd	nd	nd
1b	0.077 ± 0.03	0.440 ± 0.01[b]	1.716 ± 0.0[c]	max	max
4b	nd	nd	nd	nd	nd
1c	0.204 ± 0.07[b]	1.222 ± 0.38[c]	max	max	max
4c	0.094 ± 0.01	0.216 ± 0.03[b]	0.624 ± 0.06[c]	1.450 ± 0.15[c]	max
4d	0.081 ± 0.02	0.217 ± 0.05[a]	0.735 ± 0.08[c]	1.499 ± 0.03[c]	max
2b	0,148 ± 0.05	0.735 ± 0.16[c]	1.861 ± 0.20[c]	max	max
2c	0.165 ± 0.50[a]	0.887 ± 0.16[c]	max	max	max

Data expressed mean ± SE of absorbance at 593 nm. (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$ when compared with control sample (FRAP solution without compounds = 0.092 ± 0.02) by Student-Newman-Keuls test for post-hoc comparison. (nd): not detected. (max): maximal absorbance detected.

Inserting of Se-aryl group on **1a** structure given **4d** and **2b**, didin't change the reducing power of compounds. Interesting, the reducing capacity of **4c**, which has the substitution of *ortho*/*para*-hydroxyl groups for Se-aryl group, was higher than **1a** and lower than **1c**.

1a and analogues **1b**-**c** possessed significant activtiy on DPPH, ABTS and FRAP assays. As expected, according number and position of substituents of aromatic ring, **1a**-**c** had different antioxidant potential. **1c**, which has two hydroxyl groups as substituent on *para*/*meta*-position of aromatic ring, showed the highest scavenging activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and reducing capacity on FRAP assay**.** When IC_{50} and *n* values were compared, **1c** had 9.5 and 41 times more scavenging activity agains DPPH radicals than **1a** and **1b**, respectivelly. Against ABTS radicals, compound **1c** showed 1.3 and 1.9 times more effective than **1a** and **1b**.

On the other hand, **4b** showed lower capacity to scavenge DPPH and no effect against ABTS radicals. Only organochalcogen derivatives **4c**, **2b** and **2c**, possessed better DPPH scavenging activity than the **1a**. In addition, **4c** and **2c** showed higher ABTS scavenging activity than **1a**, as showed in Table 1. Differently, **4d** had a clear reduction of ABTS radicals scavenging potential when compared to all compounds, while **2b** exhibit similar activity than **1a**. This finding may suggest that

the better effect of Se-aryl group to stabilize phenoxyl radical of **1a** moyet is from Michael addition.

Taken together, these findings showed that Michael adducts **2b** and **2c** were better antioxidants than **1a**. Substitution of one of the OH phenolic group on *ortho*-/*para*-position for Se-aryl group (**4c**), also contributed to the free radical scavenging activtiy of compounds.

The low and insignificant free radicals scavenging activity and power reduction of compounds **4a** and **4b**, clearly show the important role of phenolic hydroxyl groups for antioxidant capacity of **1a** analogues. In this line, phenolic-OHs in **1a** and **1c** contribute the antioxidant activity, but low *n* of **1b**, indicate that phenolic-OH on *meta*-position itself does not contribute largely to the antioxidant effectiveness. On the other hand, two adjacent phenolic-OHs of **1c** increase the reducing power and radical-scavenging ability. This data are in agreement with the findings in the research of antioxidant proprierties of **1a** derivatives.[24]

A comparison of IC_{50} and *n* values of the corresponding compounds **1a**-**c** with **4a**-**d** and **2b**-**c** indicates that inserting a Se/S-aryl group on C of double bond of **1a** results in an significant increase on antioxidant potential against DPPH/ABTS radicals.

Theses findings reflect that the insertion of Se/S-aryl groups on α,β-insaturation of **1a**, could stabilize resonantly the phenoxyl radical.

NO has an important physiological regulator function, on vasodilatation, neurotransmission and defense under pathological condition. On the other hand, high concentration of NO have central role in pathologies like inflammation and immune diseases.[28] Additionally, NO can react with superoxide anion radical ($O_2^{\bullet-}$), generating peroxinitrite ($ONOO^-$) and $OH^{\bullet}$.[29] The NO generated from SNP reacts with O_2 to form nitrite anion that was restrained by **1a**-**c**, **4d** and **2c** (Table 3). According maximum inhibition (Imax) values, derivatives **4d** and **2c** presented lower NO scavenging activity than **1a** (IC_{50} = 90.5 ± 2.0), but equal to **1b** and **1c**. Our results are in agreement with Parihar et al. (2007)[25], who also showed that **1a** scavenges NO generated by SNP.

However, **1a**-**c, 2b**-**c** and **4a**-**d** compounds didn't exhibited $^{\bullet}OH$ scavenging activity at concentration tested 10-500 µM (data not show). It can be explained, by the highest reactive of $^{\bullet}OH$, which has half-life of 10^{-9} s and reacts with any cellular

component.[30] In this sense, some data have shown that •OH cannot be efficiently scavenged by some natural antioxidants (e.g. vitamins C and E, and polyphenols).[31]

Table 3 – Inhibition effect of linoleic acid peroxidation induced by SNP and NO-scavenging activty of compounds.

Compound	Linoleic acid peroxidation			NO-scavenging activity	
	IC_{50} (μM)	Imax (%)	Effective concentration	IC_{50} (μM)	Imax (%)
1a	ns	ns	ns	90.5 ± 2.0	60.50 ± 5.1
4a	ns	43.12 ± 8.4	10-500	ns	ns
1b	335.0 ± 10.0	52.73 ± 3.8	10-500	ns	24.81 ± 4.2
4b	ns	42.97 ± 8.9	10-500	ns	ns
1c	ns	48.72 ± 11.2	50-500	10-100	26.69 ± 1.8
4c	279.0 ± 10.0	62.08 ± 2.8	50-500	ns	ns
4d	ns	47.77 ± 1.0	50-500	ns	29.91 ± 4.4
2b	66.6 ± 3.6	71.59 ± 5.3	10-500	ns	ns
2c	ns	42.43 ± 8.9	100-500	ns	25.70 ± 5.4

Linoleic acid peroxidation induced by SNP

The ability of compounds to inhibit the direct lipid oxidation was evaluated on a linoleic acid emulsion. Linoleic acid oxidation generates lipid peroxides and hydroperoxides, which decompose to secondary oxidation products like malondialdehydes (MDA). In this study, the possible effects of **1a** and its analogues to decrease the lipid peroxidation was used the thiobarbituric acid-reactive substances (TBARS) assay. Table 3 shows the effect of of compounds against linoleic acid peroxidation induced by SNP. When the maximum inhibition (I_{max}) values are analised, **1b**-**c, 4a**-**d** and **2b**-**c** showed an effective protection for lipid peroxidation ranging from 42.43 to 71.59%. Only **1b**, **4c** and **2b** had the capacity to reduce more than 50% the lipid peroxidation. **2b** showed the best antioxidant capacity (IC_{50} = 66.6 ± 3.6 μM).

In comparison to NO scavenging activity data, just the the antioxidant activity of **1b**-**c**, **4d** and **2c** can be related, at least in part to ability to scavenge NO radicals generated by SNP. On the other hand, the antioxidant activity of **4c** and **2b** in this system is not related to NO itself, but probably it is by inhibit the chain reaction of lipid peroxidation by interaction with intermediates like peroxyl radicals.

RBC hemolysis induced by 2,2'-azo(2-asmidinopropane)dihydrocloride (AAPH)

Antioxidant activity of compounds in homogenous solutions, like assays discussed before, may not reproduces the effects in heterogeneous media like cells and *in vivo* experiments.[32] The antioxidant effect of **1a**-**c**, **4a**-**d** and **2b**-**c** was investigated in RBC hemolysis model to the best understanding of biological activities and to evaluate the influence of endogenous constituents on the antioxidant activity. AAPH-induced hemolysis is a convenient assay carried out to mimic erythrocytes undergoing oxidative stress. AAPH decomposes at 37 °C in aqueous solutions generating an initiating radical ($R^{\bullet}$), which can react with O_2 and generates peroxyl radicals ($ROO^{\bullet}$) and react directly with polyunsaturated lipids. RBC membranes are damage as a result of lipid peroxidation, loss of glutathione and membrane integrity leading to release of hemoglobin (hemolysis) and intracellular K^+ ions.[33]

In the absence of AAPH the RBCs were stable and little hemolysis took place within 3 h (data not shown), whereas 50 mM AAPH induced fast hemolysis after an time to half-hemolysis of 102.0 min (Table 4). Derivatives compounds **4d**, **2b**, **2c** and quercetin effectively prolonged the time to half-hemolysis and delayed it occurrence at 50 µM (Figure 4). The concentration of 10 uM was ineffective for all compounds. Furthermore, after 3h the RBC hemolysis induced by AAPH (89.67%) was effectively decreased by derivatives **4d** (61.79%), **2b** (33.48%), **2c** (52.56%) and quercertin (42.65%), as can be seen on Fig. 2. The sequence of compounds to protect erythrocytes against AAPH-induced hemolysis was **2b** = Quercetin > **2c** > **4d**. The highest time to half-hemolysis of **4d** (145.3 min), **2b** (>180 min) and **2c** (152.4 min) indicates the essential role of Se and S-aryl groups to enhance the antioxidant ability remarkably when compared to **1a**-**c**. In the more complex system of RBC lysis assay, our data suggest that the presence of electron-donating groups besides phenolic-OHs can be responsable of inhibition of oxidative damage on lipids, proteins and DNA and eventually hemolysis under oxidative stress.

On the other hand, the presence or absence of phenolic-OH groups and a substitution one group for Se-aryl group on aromatic ring do not confer antioxidant activity for **1c** and **4a**-**c**. However, the antioxidant activity of hydroxybenzalacetones, including **1a** and **1c**, against lipid peroxidation induced by $ONOO^-$ in RBC was

showed by Motohashi et al. (2005). Authors have suggested that OH group on *ortho*- or *para*-position of the phenolic OH group had a significant effect to decrease lipid peroxidation. Additionally, 3,5-methoxy substituents had an additive antioxidant effect .[34] It can explain, at least in part, the greater anti-hemolysis effect of derivatives **4d**, **2b** and **2c** when compared to **1a**-**b**. **4d**, **2b** and **2c** have 4-OH and 3-methoxy groups on aromatic ring and the addition of electron-donating groups (-SeC_6H_5; -SC_6H_5) in different position of basic structure of **1a**. These finding suggests that the presence of chalcogen groups have an additive and essential role to improve the capacity of hydroxybenzalacetones to inhibit RBC hemolysis induced by oxidative damage.

Table 4 – Time to half-lysis red blood cells of mice by studied compounds

Sample	**Time to 50% hemolysis (min)** (Mean ± SE)	
AAPH	102.0 ± 1.7	
	10 µM	**50 µM**
1a	110.3 ± 6.1	108.6 ± 3.8
4a	96.69 ± 3.4	133.3 ± 8.9
1b	110.3 ± 1.9	111.5 ± 3.8
4b	94.8 ± 2.8	98.71 ± 2.8
1c	105.4 ± 4.2	124.4 ± 12.4
4c	107.5 ± 2.9	123.7 ± 6.0
4d	113.1 ± 2.9	145.3 ± 10.8 **
2b	104.2± 6.3	> 180 ***
2c	111.4 ± 6.9	152.4 ± 12.87 ***
Quercetin	119.8 ± 3.0	> 180 ***

(*) denote $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ when compared to AAPH sample Student-Newman-Keuls test for post-hoc comparison.

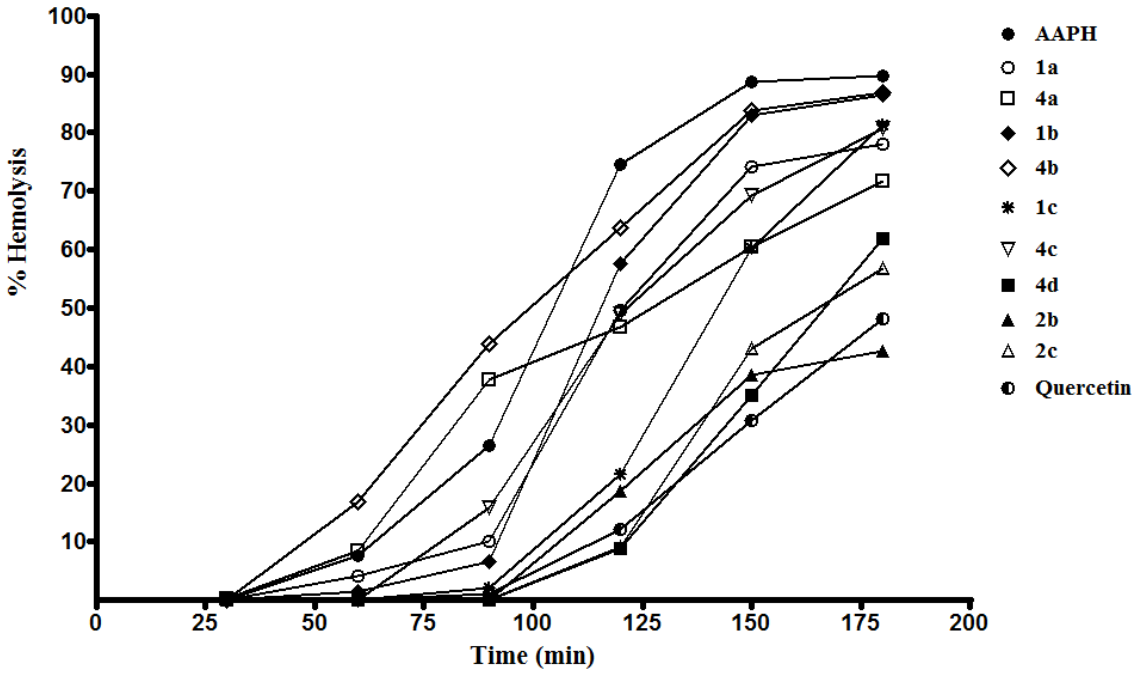


Fig. 2 – Effect of compounds and quecertin (50 µM) on 50 mM AAPH-induced hemolysis of mice RBCs in PBS (pH 7.4) under air atmosphere at 37 °C.

Conclusions

In conclusion, we have examined the antioxidant ability of a series dehydrozingerone analogues. Some derivatives compounds showed enhanced DPPH-scavenging, lipid peroxidation inhibition and anti-hemolysis activities, being the Michael adducts containing Se and S-aryl groups the more actives. This could be a strategy for the development of new semi-synthetic antioxidants.

EXPERIMENTAL SECTION

General Information: Reactions were monitored by TLC carried out on Merck silica gel (60 F_{254}) by using UV light as visualizing agent and 5% vanillin in 10% H_2SO_4 and heat as developing agents. Baker silica gel (particle size 0.040-0.063 mm) was used for flash chromatography. Hidrogen nuclear magnetic resonance spectra (^{1}H NMR) were collected at 300 MHz on a Varian Gemini NMR and at 400 MHz on Bruker DPX 400 spectrometers. Spectra were recorded in $CDCl_3$ solutions. Chemical shifts are reported in ppm, referenced to tetramethylsilane (TMS) as the external reference. Hydrogen coupling patterns are described as singlet (s), doublet

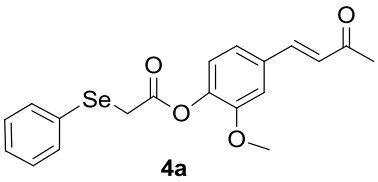
(d), triplet (t) and multiplet (m). Coupling constants (*J*) are reported in Hertz. Carbon-13 nuclear magnetic resonance spectra (^{13}C NMR) were obtained at 75 MHz on a Varian Gemini NMR and at 100 MHz on Bruker DPX 400 spectrometers. Chemical shifts are reported in ppm, referenced to the solvent peak of $CDCl_3$. Low-resolution mass spectra (LRMS) were obtained with a Shimadzu GC-MS-QP2010 mass spectrometer. GC analysis were conducted on a RESTEC RTX-5MS capillary column (30 m, 0.25 mm id, 0.25 µm film thickness) using the products dissolved in ethyl acetate with the following conditions: Injected sample volume was 1.0 µL; He constant flow, 54.1 mL/min; initial inlet temperature, 40 °C ramped to 72 °C at 10 °C/min followed by a 5 °C/min ramp to 100 °C (held for 10 min) and 10 °C/min to 280 °C and held for 20 min (total run time: 56.8 min). Infrared spectra (□max) were recorded on a Bruker Vector 22 Fourier-Transform Spectrometer or a Perkin Elmer 1720-X FT-IR Spectrometer. Samples were analyzed using KBr Diffuse Reflectance Fourier Transform (DRIFT) spectra for solids. High-resolution mass spectra (HRMS) were obtained for all compounds on a LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific). This hybrid system meets the LTQ XL linear ion trap mass spectrometer and an Orbitrap mass analyzer. The experiments were performed via direct infusion of sample (flow: 10 µL/min) in the positive-ion mode using electrospray ionization. Elemental composition calculations for comparison were executed using the specific tool included in the Qual Browser module of Xcalibur (Thermo Fisher Scientific, release 2.0.7) software. DHZ (**1a**)[5i], *iso*-DHZ (**1b**)[13a] and 3,4-dihydroxybenzalacetone (**1c**)[13b], 2-(phenylselanyl)acetic acid (**5**)[14] and 2-(phenylselanyl)acetyl chloride[15] were synthesized according described in literature; spectral data and mp of synthesized compounds were in perfect agreement with those previously described.

Synthesis of compounds, spectral and analytical data.

Synthesis of derivatives 4a and 4b. Under N_2 atmosphere, phenylselanylacetic acid (1.27 mmol, 0.273g) was dissolved in dichloromethane (7 mL) and catalytic amount of DMAP was added. Then DCC (1.39 mmol, 0.286g) was added at 0 °C. After 10 min, DHZ (**1a**) or *iso*-DHZ (**1b**) (0.192g, 1.0 mmol) was added portion wise and the reaction mixture was stirred for additional 10 min at 0 °C. After that, the

reaction was allowed to react at room temperature. After the time correspondent to each product (**4a**, 24h; **4b**, 1.5 h), the reaction was quenched with water (20 mL) and extracted with dichloromethane (3x 15 mL), dried ($MgSO_4$) and concentrated at low pressure. The purification by chromatography column was carried out using a mixture of ether:ethyl acetate (97:3) as the eluent.

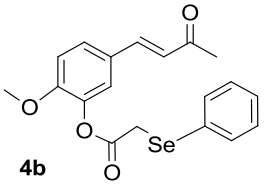
(*E*)-2-methoxy-4-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate (4a):



4a

Yield: 0.058g (15%); pale yellow solid; mp 78-79 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.68-7.66 (m, 2H), 7.46 (d, *J* = 16.4 Hz, 1H), 7.32-7.30 (m, 3H), 7.12-7.10 (m, 2H), 6.96 (d, *J* = 8.8 Hz, 1H), 6.65 (d, *J* = 16.4 Hz, 1H), 3.83 (s, 3H), 3.75 (s, 2H), 2.38 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 198.2, 168.6, 153.0, 142.6, 141.5, 133.5, 133.5, 129.3, 129.2, 128.0, 127.3, 123.0, 121.4, 111.4, 55.9, 27.5, 26.9. IR (KBr pellet), ν (cm^{-1}): MS (relative intensity) *m/z*. HRMS (ES) for $C_{19}H_{18}O_4Se$+Na ($[M^+Na]^+$): calcd: 413.0263; found: 413.0263. ^{77}Se NMR ($CDCl_3$, 95.4 MHz): δ = 341.27 (t, *J* = 14.3 Hz).

(*E*)-2-methoxy-5-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate (4b):

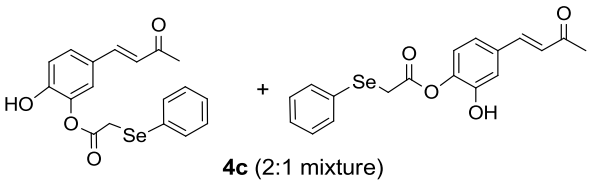


4b

Yield: 0.226g (58%); pale yellow solid; mp 103-105 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.69-7.66 (m, 2H), 7.40 (d, *J* = 16.0 Hz, 1H), 7.36 (dd, *J* = 8.4 and 2.0 Hz, 1H), 7.32-7.29 (m, 3H), 7.09 (d, *J* = 2.0 Hz, 1H), 6.94 (d, *J* = 8.4 Hz, 1H), 6.54 (d, *J* = 16.0 Hz, 1H), 3.81 (s, 3H), 3.75 (s, 2H), 2.34 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 198.1, 168.7, 153.0, 142.2, 139.9, 133.6, 133.5, 129.3, 129.2, 128.1, 125.9, 121.8, 112.5, 55.9, 27.5, 26.9. HRMS (ES) for $C_{19}H_{18}O_4Se$+Na ($[M^+Na]^+$): calcd: 413.0263; found: 413.0263. . ^{77}Se NMR ($CDCl_3$, 95.4 MHz): δ = 342.24 (t, *J* = 14.3 Hz).

Synthesis of derivatives 4c: To a solution of 3,4-dihydroxybenzalacetone (**1c**) (0.875 mmol, 0.156 g) in DMF (2.5 mL) at 0 ^{o}C under N_2 atmosphere, imidazole (0.875 mmol, 0.060g) was added under stirring. Then, a solution of phenylselanyl acetyl chloride ($PhSeCH_2COCl$, .875 mmol, 0.204g) in DMF (2.5 mL) was added dropwise. After 10 min at 0 ^{o}C, stirring was continued at room temperature for additional 18.5 h. Then, the crude mixture was received in water (15 mL) and extracted with diethyl ether (3x 10 mL). The organic layer was washed with water (15 mL) and brine (15 mL). The organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. After purification by HPLC, product was obtained as a 2:1 mixture of monoacylated compounds (*E*)-2-hydroxy-4-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate and (*E*)-2-hydroxy-5-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate, called **4c**. Yield: 0.053g (14%); yellow solid; mp 97-99 °C.

(*E*)-2-hydroxy-5-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate (major isomer) and (*E*)-2-hydroxy-4-(3-oxobut-1-en-1-yl)phenyl 2-(phenylselanyl)acetate (minor isomer) (4c):

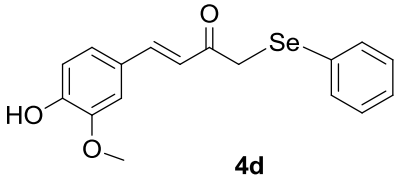


4c (2:1 mixture)

^{1}H NMR ($CDCl_3$, 400 MHz), major isomer: δ = 7.66-7.63 (m, 2H), 7.41 (d, *J* = 16.0 Hz, 1H), 7.36-7.27 (m, 4H), 7.18 (d, *J* = 2.0 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.54 (d, *J* = 16.0 Hz, 1H), 3.72 (s, 2H), 2.35 (s, 3H). minor isomer: δ = 7.66-7.63 (m, 2H), 7.41 (d, *J* = 16.4 Hz, 1H), 7.36-7.26 (m, 4H), 7.15 (d, *J* = 1.6 Hz, 1H), 7.06-7.04 (m, 1H), 6.62 (d, *J* = 16.4 Hz, 1H), 3.72 (s, 2H), 2.35 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz), mixture 4c: δ 198.9, 198.8, 169.1, 169.0, 149.8, 147.9, 142.8, 142.7, 139.7, 138.2, 133.8, 133.7, 133.5, 129.6, 129.5, 129.3, 128.7, 128.6, 128.1, 128.0, 127.8, 127.4, 127.3, 125.7, 122.9, 122.3, 121.0, 117.8, 116.6, 27.5, 27.4, 27.3, 27.2. HRMS (ES) for $C_{19}H_{18}O_4Se$+Na ($[M^+Na]^+$): calcd: 377.0287; found: 377.0287. . ^{77}Se NMR ($CDCl_3$, 95.4 MHz): δ = 354.14 (t, *J* = 15.3 Hz) e 353.85 (t, *J* = 15.3 Hz).

(*E*)-4-(4-hydroxy-3-methoxyphenyl)-1-(phenylselanyl)but-3-en-2-one (4d):[16]

To a mixture of DHZ (1a) dehydrozingerone (0.384g, 2.0 mmol), diphenyl diselenide (0.312 g; 1.0 mmol) in PEG-400 (2 mL) under N_2, KF/Al_2O_3 (0.64 g) was added at room temperature. The temperature was increased to 60 ºC and mixture was stirred for 22 h. The product was extracted with petroleum ether (3x 5 mL) and the organic phase dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography over flash silica gel eluting with petroleum ether: acetyl acetate (98:2). The 1-phenylseleno DHZ **4d** was obtained as a yellow oil.

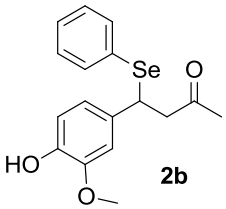


4d

Yield: 0.070g (20%). yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.92-7.49 (m,2H), 7.35 (d, *J* =15.9 Hz, 1H), 7.22-7.19 (m, 3H), 6.95 (dd, *J* = 8.2 and 1.9, 1H), 6.89 (d, *J* = 1.9 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.59 (d, *J* = 15.9 Hz, 1H), 5.87 (bs, 1H), 3.92 (*J* = 5.3, 1H), 3.79 (s, 2H). HRMS (ES) for $C_{19}H_{18}O_4Se$+Na ($[M^+Na]^+$): calcd: 413.0268; . ^{77}Se NMR ($CDCl_3$, 95.4 MHz): δ = 321.89 (t, *J* = 14.3 Hz).

4-(4-hydroxy-3-methoxyphenyl)-4-(phenylselanyl)butan-2-one (2b):[19a]

To a solution of diphenyl diselenide (0.312 g, 1.0 mmol) in ethanol (2.5 mL) under N_2 atmosphere, $NaBH_4$ (0.049g, 1.3 mmol) was added at room temperature and the mixture stirred for 30 min. The colorless (or faint yellow) solution of bezeneselenolate obtained was cooled to 0 °C in an ice bath and acetic acid (2.3 mmol) was added. Following, a solution of DHZ **1a** (0.192 g, 1.0 mmol) in ethanol (0.5 mL) was added and the resulting mixture was stirred at 0 °C for 4 h. After consuming the starting materials (TLC), the mixture was diluted with ethyl acetate (15 mL) and washed with water (15 mL) and brine (3 x 15 mL). The organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using ethyl acetate/hexane (30% v/v) as the eluent, yielding the product as a pale yellow solid. Spectral data are depicted below.

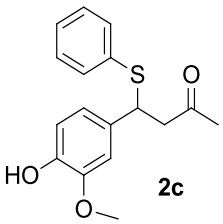


2b

Yield: 0.056g, 16%, mp 82-85 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.48-7.19 (m, 5H), 6.93-6.56 (m, 3H), 5.77 (br s, 1H), 4.73 (dd, *J* = 6.3 and 8.7 Hz, 1H), 3.75 (s, 3H), 3.19 (dd, *J* = 8.7 and 16.9 Hz, 1H), 3.02 (dd, *J* = 6.3 and 16.9 Hz, 1H), 2.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.8, 146.2, 144.8, 134.0, 132.9, 132.7, 128.8, 127.5, 120.2, 114.2, 110.4, 55.8, 49.7, 47.9, 30.7. IR (KBr pellet), ν (cm^{-1}): 1276.9, 1514.1, 1689.6, 2968.4, 3361.9. MS (relative intensity) *m/z*: 194 (M^+-SePh, 5.5), 193 (43.9), 192 (34.5), 177 (35.8), 158 (26.5), 151 (15.9), 150 (21.4), 145 (63.1), 135 (18.4), 117 (28.0), 111 (12.3), 109 (11.3), 97 (23.2), 95 (19.3), 83 (25.3), 81 (26.3), 78 (70.6), 69 (38.7), 67 (17.9), 57 (40.5), 55 (37.7), 43 (100). HRMS (ES) for $C_{17}H_{18}O_3Se$+Na ($[M^+Na]^+$): calcd: 373.0313; found: 373.0909.

4-(4-hydroxy-3-methoxyphenyl)-4-(phenylthio)butan-2-one (2c):[18c]

To a round-bottomed flask containing mixture of benzenephenol (0.065g, 0.6 mmol) and KF/Al_2O_3 (0.07 g) in glycerol (1.0 mL) was added DHZ (**1a**, 0.058g, 0.3 mmol). The reaction mixture was stirred at 90 °C for 4 h under N_2 atmosphere. After completion of the reaction (TLC), the mixture was cooled to room temperature, diluted with ethyl acetate (15 mL) and washed with aq. NH_4Cl (3 x 15 mL). The solvent was evaporated under reduced pressure and the residue was purified by column chromatography over silica gel eluting with hexane/ethyl acetate (20% v/v) yielding the product as a white solid.



2c

Yield: 0.078g, 86%. White solid, mp 78-80 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.34-7.24 (m, 5H), 6.64-6.77 (m, 3H), 5.71 (br s, 1H), 4.68 (dd, *J* = 6.6 and 8.1 Hz, 1H), 3.83 (s, 3H), 3.08 (dd, *J* = 16.8 and 8.1 Hz, 1H), 3.00 (dd, *J* = 16.8 and 6.6 Hz, 1H),

2.09 ppm (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 205.9, 146.4, 144.9, 134.1, 133.0, 132.8, 128.9, 127.6, 120.3, 114.3, 110.5, 55.9, 49.7, 48.0, 30.8. IR (KBr pellet), ν (cm^{-1}): 1282.7, 1515.1, 1689.7, 2960.8, 3362.9. MS (relative intensity) *m/z*: 302 (M^+, 0.2), 194 (5.1), 193 (42.0), 177 (2.0), 151 (10.6), 145 (3.0), 135 (5.5), 119 (1.4), 110 (5.5), 107 (2.4), 91 (2.1), 77 (4.3), 65 (3.1), 43 (100.0). HRMS (ES) for $C_{17}H_{18}O_3S^+Na$ ($[M^+Na]^+$): calcd: 325.0869; found: 325.0874.

Spectral data (^{1}H and ^{13}C NMR) of compounds **2b**, **2c** and **4d** are presented in Appendix A.

Antioxidant activity assays

To evaluate the antioxidant activity of different concentrations, all compounds were diluted in dimethylsulfoxide (DMSO) at concentrations ranging from 10-500 μM. For anti-hemoysis assay the comounds were tested at 10 and 50 μM. The results are expressed as the mean ± standard error (S.E.) pf the minimal three repetitions and the data were validated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test, when appropriate. The IC_{50} values (concentration providing 50% effect) were calculated from the graph of effect percentage *versus* fruits concentration; maximal inhibition (I_{max}) values were calculated at the most effective concentration used. The effects of the compounds on hemolysis are expressed as time to 50% hemolysis calculated by sigmoidal curve-fitting of absorbance curves using Prism 5 (GraphPad, La Jolla, CA, USA) and % of hemolysis *versus* time. A *p* value less than 0.05 ($p < 0.05$) was considered significant by.

Diphenyl-2-picryl- hydrazyl (DPPH) radical-scavenging assay

Measurement of the scavenging activity of the organochalcogen compounds against the DPPH$^\bullet$ was performed in accordance with the procedure reported by Choi (2002)[35]. Briefly, 50 µM of DPPH$^\bullet$ in ethanol was added to medium containing different concentrations of compounds (1-500 µM) and incubated for 30 min at 30 °C. The absorbance of the resulting solutions and the blank were recorded spectrophotometrically at 517 nm. The results were expressed as DPPH$^\bullet$ scavenging (%) by and calculated from the following equation:

$$DPPH^{\bullet} \text{ scavenging } (\%) = (A_c - A_s / A_c) \times 100,$$

where A_c is the absorbance of the control reaction mixture excluding the test compounds and A_s is the absorbance of the reaction mixture with different concentrations of compounds. The stoichiometry (the equivalent of $DPPH^{\bullet}$ scavenged by one equivalent of antioxidant) *n* was calculated follow the equation:

$$n = 50\ (\mu M)/\ (IC_{50}\ (\mu M) \times 2)$$

2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$) scavenging activity

$ABTS^{\bullet+}$ scavenging activity of compounds was carried out according method described by Re (1999)[22], that involves the oxidation of ABTS aqueous solution (7 mM) with potassium persulfate (2.45 mM). The reaction mixture is stored in the dark at room temperature for 12-16 hours. Before usage, the $ABTS^{\bullet+}$ solution was diluted in PBS (0.1 M, pH 7.4) resulting in a solution of 103 µM . The assay start when 990 µL of $ABTS^{\bullet+}$ solution was added to 10 µL of compounds at different concentrations (0.5-500 µM) and stayed for 30 minutes at room temperature. The results were expressed as percentages of decrease of $ABTS^{\bullet+}$ concentration by decrease of absorbance values and calculated from the following equation:

$$ABTS^{\bullet+} \text{ scavenging } (\%) = (A_c - A_s/A_c) \times 100\ ,$$

where A_c is the absorbance of the control sample ($ABTS^{\bullet+}$ solution excluding the test compounds) and A_s is the absorbance of the reaction mixture with test compounds in different concentrations. The stoichiometry (the equivalent of $ABTS^{\bullet+}$ scavenged by one equivalent of antioxidant) *n* was calculated follow the equation:

$$n = 103\ (\mu M)/\ (IC_{50}\ (\mu M) \times 2)$$

Hydroxyl radical ($OH^{\bullet}$) scavenging activity

Hydroxyl radical-scavenging activity of compounds was determined according to the method described by Smirnoff and Cumbes (1989)[36] with some modifications. The assay was carry out through with the reaction of 100 µL of sodium salicylate (20 mM), 100 µL of $FeSO_4$ (1.5 mM), 10 µL of compounds at different concentrations (10-500µM) and started with addition of 200 µL of H_2O_2 (6mM) (the total volume was adjusted to 1000 µL with distilled water). The reaction mixture was allowed in water bath for 1h at 37 ºC and the absorbance of was recorded at 510 nm. The results

were expressed as percentages of decrease of $OH^{\bullet}$ formed by decrease of absorbance values and calculated from the following equation:

$$OH^{\bullet} \text{ scavenging activity (\%)} = (A_c - A_s/A_c) \times 100$$

where A_c is the absorbance of the control sample (reaction mixture in absence of compounds) and A_s is the absorbance of the reaction mixture with test compounds in different concentrations.

Nitric oxide (NO) scavenging activity

NO scavenging activity of compounds was measured according to the method of Marcocci et al. (1994)[37]. 10 µl of compounds at different concentrations (10-100 µM) were mixed to 990 µL of sodium nitroprusside (SNP) solution (25 mM). The reaction mixture was allowed during 2h under light at 37 °C. An aliquot (0.25 mL) of the sample was removed and diluted in 0.25 mL of Griess reagent. After 5 minutes, the absorbance of the chromophore (formed during the diazotiation of nitrite with sulfanilamide and its subsequent coupling with napthylene diamine) was read at 570 nm. Percentage scavenging was calculated using the equation :

$$\text{NO scavenging (\%)} = (A_c - A_s/A_c) \times 100,$$

where A_c is the absorbance of the control sample (reaction mixture in absence of tested compouds) and A_s is the absorbance of the reaction mixture with different concentration of compounds.

Ferric ion reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was measured according to the method described by Stratil (2006)[38] with slight modifications. The FRAP reagent was prepared by mixing aqueous solutions of sodium acetate buffer (38 mM, pH 3.6), $FeCl_3 \cdot 6H_2O$ (20 mM) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (10 mM in 40 mM of HCl) in a 10:1:1 ratio. This reagent was freshly prepared before each experiment. To each sample, 10 µL of compounds at different concentrations (1-500 µM) and 990 µL of the FRAP reagent were added, and the mixture was incubated for 40 min in the dark at 37 ºC. The absorbance of the resulting solution was recorded at 593 nm. Results were compared to control sample (FRAP solution eithout compounds) and expressed in absorbance values.

Linoleic acid peroxidation induced by SNP

The ability of compounds to inhibit the lipid peroxidation was performed using a linoleic acid emulsion system, according the method described by Choi et al. (2002)[35], with modifications. All reagents were prepared fresh. Reaction mixture contained 250 µL linoleic acid emulsion (48.8 mM and SDS 0,025%), 10 µL of of compounds (10-500 µM), 50 µL $FeSO_4.7H_2O$ (48.8 mM) and volume adjusted with Tris-HCl (100 mM, pH 7.5) for 610 µL . Linoleic acid peroxidation was initiated by the addition of 10 µL of NPS (12.2 mM). The mixture was incubated for 30 min at 37 °C and stopped by the addition of 125 µL trichloroacetic acid (5.5%). An aliquot of 250 µL of reaction mixture was added to 250 µL of thiobarbituric acid (1% in 50 mM NaOH), and then placed in a water bath at 90 °C for 10 min. After incubation, the reaction was stopped by placing in an ice-bath for 10 min and 600 µL *n*-butanol were added into the reaction mixture and mixed vigorously. The organic phase was separated after centrifuge at 3000 g for 5 min. The absorbance of sample (organic phase) was measured at 532 nm using *n*-butanol as blank. The results was expressed in percentage of lipid peroxidation, using the induced sample (emulsion system with NPS in absence of compounds) as 100% lipid peroxidation.

Erythrocyte preparation

Male Swiss mice (30 ± 5 g mean body weight) were anaesthetised and blood (0.5 ml) was obtained via cardiac puncture using a heparinised 1 ml syringe and 25G needle. As described by Sekiya et al. (2002)[39], erythrocytes were suspended in ice cold phosphate-buffered saline (PBS, Aldrich in mM, NaCl 137; KCl 2.7; Na_2HPO_4 10 and NaH_2PO4 2, pH 7.2-7.6) using 0.9 µL of PBS for every 1 µL of blood and separated from plasma by centrifugation at 2600 g for 10 min at 4°C. The supernatant and buffy coat layers were removed via pipetting and the packed erythrocytes were resuspended as before and centrifuged again for 5 min at 1500 g (Sekiya et al., 2002). This stock of erythrocytes was stored as a packed cell mass at 4°C until analysis. The working suspension of erythrocytes were prepared by diluting 100 µL of the stock with 5 mL of cold sterile PBS.

RBC hemolysis induced by AAPH

The hemolysis assay was performed according Jani et al. (2012)[40] with some modifications Erythrocyte suspension (50 µL) was incubated in a 96 well plate with reduced glutathione (20 µL , 1mM) and 10 or 50 mM of the compounds for 30 min. Following this incubation period,the water-soluble free radical generator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH, 150 mM) was added to each of the wells and lysis immediately measured using a spectrophotometer (ThermoElectron Corporation,Vantaa,Finland) at 630 nm wave length. Absorbance readings of the wells were taken every 30 min for 3h. For 100% hemolysis, cells were pre-incubated with 150 µL of miliQ water. The % hemolysis during incubation time was calculed by equation:

$$\% \text{ hemolysis} = [(A_s - A_c)/(A_{100} - A_0)] \times 100,$$

where A_s is the absorbance of sample, A_c is the absorbace of control (PBS control), A_{100} the absorbance of 100% hemolysis (H_2O-induced).

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4 Pesquisa exploratória sobre a capacidade antioxidante, antimicrobiana e toxicidade do óleo essencial das folhas de *Campomanesia xanthocarpa* (guabiroba)

A partir da revisão da literatura foi observado que a parte vegetativa da espécie *C. xanthocarpa* apresenta várias propriedades farmacológicas atribuídas aos compostos do seu metabolismo secundário. Devido à escassez de dados referentes ao óleo essencial das folhas, este trabalho buscou complementar os estudos e avaliar pela primeira vez sua capacidade antioxidante, antimicrobiana e toxicidade. O manuscrito científico foi submetido ao periódico *Pharmaceutical Biology,* ISSN: 1744-5116.

Essential oil of *Campomanesia xanthocarpa* leaves: A screening of antioxidant, antimicrobial and toxicological properties

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Abstract

Context: The effect of *Campomanesia xanthocarpa* Berg. leaves extracts has been evaluated in hypercholesterolemia, gastric ulceration and diabetes conditions.

Objective: This study screens the *in vitro* antioxidant and antimicrobial activities and the *in vivo* toxicity of essential oil (EO of *C. xanthocarpa* leaves.

Materials and methods: The antioxidant capacity was determined by ferric ion reducing antioxidant power (FRAP), chelating Fe^{2+}, 2,2-diphenyl-2-picrylhydrazyl (DPPH) and azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activities, and lipid peroxidation inhibition detected by tiobarbituric acid reactive species (TBARS) assay. Antimicrobial activity was tested using broth microdilution and agar disc diffusion method against seven bacterial and seven fungal strains. Swiss male mice were treated with a single dose of EO (100 and 500 mg/kg) 72 h before biochemical analysis.

Results: The EO presented a significant effect in FRAP (from 1000 µg/ml), DPPH (IC_{50} =1710.0 ± 5.3 µg/ml), and ABTS (IC_{50} = 1980 ± 6.3 µg/ml) assays. The lipid peroxidation of linoleic acid (IC_{50} = 202.0 ± 5.4 µg/ml), liver (IC_{50} = 1960.0 ± 6.2 µg/ml), kidney (I_{max} = 21.0 ± 6.0%), brain (I_{max} = 29.5 ± 2.0%), hippocampus (IC_{50} = 64.5 ± 8.0 µg/ml), cortex (IC_{50} = 40.0 ± 6.2 µg/ml) and cerebellum (IC_{50} = 52.0 ± 4.5 µg/ml) were reduced by EO. Significant antibacterial (MIC = 31.2 - 125 µg/ml) and antifungal (MIC = 125 - 250 µg/ml) activities were observed. No toxic effect was observed in mice after acute treatment.

Conclusions: *C. xanthocarpa* leaves EO has antioxidant and antimicrobial properties and does not cause toxicity in mice at tested doses.

Keywords: lipid peroxidation, free radical scavenger, antibacterial, antifungal.

Introduction

Natural compounds, as essential oils (EOs) extracted from plants, have attracted special attention due their important biological activities (Ruberto & Baratta, 2000). EOs are volatile, natural complex mixtures of secondary metabolites that usually are concentrated in the bark, leaves and fruits (Bakali et al., 2008). For many years, they have been widely used as fragrance and flavoring agents in food and

beverages, but EOs from several species have received additional attention by pharmaceutical and food industries due to newly discovered functions, especially anticancer (Bakali et al., 2008), antioxidant, and antimicrobial activities (Sachetti et al., 2005; Xu et al., 2013; Pirbalouti et al., 2014).

In the pharmaceutical industry, EOs have been used as medicines or coadjutants. In Brazil, successful applications include the EO from *Cordia verbenacea* DC. (Boraginaceae), an anti-inflammatory medicine for topical use (Acheflan®), and clove oil (*Syzygium aromaticum* L. Merr. & L.M. Perry, Myrtaceae), which is widely used in dental care as a sealing component and antiseptic for oral hygiene.

The edible plant *Campomanesia xanthocarpa* Berg. (Myrtaceae) is present in southern Brazil, Argentina, Paraguay and Uruguay. C. xanthocarpa leaves are used locally for weight loss, and the potential control of obesity-related symptoms, including hyperlipidemia, has been studied (Dickel et al., 2007). In addition, many studies have demonstrated pharmacological effects from extracts of C. xanthocarpa leaves: reducing total blood cholesterol and LDL levels in hypercholesterolemic patients (Klafke et al., 2010); prevention of gastric ulceration induced by ethanol in rats (Markman et al., 2004); decreasing blood glucose levels in diabetic rats (Vinagre et al., 2010) and antiplatelet, antithrombotic and fibrinolytic activities in mice (Klafke et al., 2012).

To the best of our knowledge, no study of the biological properties of the EO from *C. xanthocarpa* leaves has yet been reported. Based on these considerations, the present study evaluated the in vitro antioxidant and antimicrobial capacities, and the acute toxicity in mice of the EO from *C. xanthocarpa* leaves. These findings are relevant to possible future applications of the EO as a novel source of phytochemicals with biological activity.

Materials and methods

Chemicals

2,2ˈ-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tripyridyltriazine (TPTZ)

were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Buffer salts and all other chemicals were of analytical grade.

Animals

Twenty six male adult Swiss mice (25-35 g) were used for *ex vivo* experiment for acute toxicity evaluation. Animals were kept in a separate animal room under a 12 h light/12 h dark cycle at 22 ± 2°C with free access to food and water. Six mice were used to *in vitro* lipid peroxidation assay with tissue homogenates. All procedures were performed in accordance with institutional policies related to the handling of experimental animals (approved by the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil, process CEEA 10329).

Plant material, extraction and chemical characterization of essential oil

C. xanthocarpa leaves were collected in Frederico Westphalen city, Rio Grande do Sul (Brazil), in May 2011. The plant material was identified and botanically authenticated by Msc. M. M. Marchi, with a voucher specimen (M.M.Marchi 4389) in Herbarium of Embrapa (HECT, Pelotas-RS). Leaves were air-dried at room temperature for five days, without direct exposure to sunlight. The EO was obtained by steam distillation for 4 h using a portable oil distillation apparatus (Model: Linax-D1) followed by extraction with hexanes. The hexanic EO solution was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure at 25°C. The EO percentage was expressed as w/w (in relation to the weight of dried leaves). The EO was stored in sealed, dark vials at 4°C until experiments.

Identification of the chemical constituents of the EO was performed using Gas Chromatography coupled with Mass Spectrometry (GC-MS) analysis. The EO was diluted in hexanes, and the injected sample volume was 1.0 µl. A Shimadzu GC-MS QP2010 and a DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) were used for the analysis. The temperature was maintained at 40°C and then raised to 250°C (10 min, 20°C/min). The carrier gas was N_2 at a flow rate of 3 ml/min. The components of the EO were identified through comparison of retention index and mass spectra to those found in the literature. The percentage composition of each

individual component was obtained from electronic integration using EI (electron ionization) at 280ºC.

Antioxidant capacity

The EO was diluted in dimethylsulfoxide (DMSO) at different concentrations (5 -2000 μg/ml) for evaluation in several assays. The free radical scavenging activity of EO was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) assays as described by Choi et al. (2002) and Re et al. (1999), respectively. The values are expressed as percentages of DPPH and ABTS radical inhibition compared to the control values found with DMSO alone, as calculated from the following:

$$\% \text{ scavenging} = [(A_c - A_s / A_c) \times 100]$$

where, A_c is the absorbance of the control reaction and A_s is the absorbance of the sample under analysis.

The ferric ion reducing antioxidant power (FRAP) and the ferrous ion (Fe^{2+}) chelating property were measured as described by Stratil et al. (2006). The values of FRAP assay were expressed in absorbance at 593 nm and the ferrous chelating effect was calculated using the following:

$$\text{Ferrous ion chelating effect (\%)} = (1 - A_s / A_c) \times 100$$

where A_c = absorbance of control, and A_s = absorbance in the presence of the EO.

The linoleic acid peroxidation assay was performed using the method of Choi et al. (2002), with modifications. The absorbance values were converted into percentage antioxidant capacity using the following*:*

$$\text{Linoleic acid peroxidation inhibition (\%)} = (A_c - A_s) \times 100 / (A_c - A_n)$$

where A_c = absorbance of control; A_s = absorbance of sample with EO; A_n = absorbance of blank (without EO, $FeSO_4.7H_2O$ and ascorbic acid).

Antioxidant assays in vitro with tissue homogenates

Swiss mice were euthanized by cervical dislocation, and the brain, liver and kidneys were rapidly removed and placed on ice. Organs were homogenized in 50 mM Tris-HCl at pH 7.4 at a concentration of 1/10 w/v, except for the brain, which was homogenized in a 1/5 w/v solution. The homogenate was centrifuged for 10 min at 2400 g to yield a pellet (discarded) and a low-speed supernatant (S_1) for each tissue, which was used for the lipid peroxidation and *δ*-aminolevulinate dehydratase (δ-ALA-D) activity assays.

The lipid peroxidation levels was evaluated by thiobarbituric acid reactive species (TBARS) assay. This assay was carried out to determine whether the EO protects against *in vitro* lipid peroxidation induced by Fe^{2+}-EDTA in liver, kidney and brain homogenates of mice. TBARS were determined as described by Ohkawa et al. (1979). Sodium nitroprusside (SNP, 25 mM) was used as an inducer of lipid peroxidation in brain regions. The hippocampus, cortex and cerebellum of mice were homogenized in cold 50 mM Tris-HCl (pH 7.4) in a proportion of 1/5 (w/v). All results are reported as % inhibition of lipid peroxidation against the control induced (with SNP, without EO = 100% lipid peroxidation).

Pro-oxidant effect on *δ*-aminolevulinate dehydratase (δ-ALA-D) activity

In vitro hepatic, renal and cerebral δ-ALA-D activities were determined by measuring the rate of product (porphobilinogen) formation, except that 84 mM PBS (pH 6.4) and 2.5 mM aminolevulinic acid were used (Sassa, 1982). Pre-incubation time with EO was 10 min (37 °C) for all tissue, and more 30 min (liver), 60 min (kiney) or 180 min (brain) at 37 °C after addition of aminolevulinic acid. The reaction product was determined using modified Ehrlich's reagent at 555 nm.

Acute toxicity in mice

Acute toxicity was assessed using the following procedure: a group of six mice received a single oral dose of the EO (100 and 500 mg/kg) diluted in canola oil and a control group received a single dose of canola oil (10 ml/kg). After administration, animals were observed for 72 h to determine the potential lethality of acute treatment. Body weight gain was recorded as a sign of general toxicity. The clinical

symptoms observed were seizures, respiratory depression, alopecia, skin lesions and diarrhea. After 72 h of exposure, animals were euthanized and the liver, kidney and brain tissue homogenates ($S_{1,}$ prepared according antioxidant assays *in vitro* with tissue homogenate (see section previous) were used for *ex vivo* analysis of lipid peroxidation (TBARS), ascorbic acid levels, catalase and δ-ALA-D activities.

Ex vivo assays

Lipid peroxidation was measured by determination by thiobarbituric acid reactive substances (TBARS assay) as previously described for antioxidant assays *in vitro* with tissues homogenates without pre-incubation time. Catalase activity was assayed spectrophotometrically by monitoring the disappearance of H_2O_2 in the presence of homogenate (S_1) at 240 nm (Aebi et al., 1995). The δ-ALA-D activity was assayed by the method described in section "*Pro-oxidant effect on δ-aminolevulinate (δ-ALA-D) activity"* without pre-incubation time. Ascorbic acid levels were determined as described by Jacques-Silva et al. (2001).

Antimicrobial activity

The following bacterial strains were used in all antimicrobial assays: Listeria *monocytogenes* 138 ATCC 19117, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* 139 ATCC 27664, *Shigella dysenteriae* ATCC 13313,

Escherichia coli ATCC 8739, *Aeromonas hydrophila* IOC 11036 and *Pseudomonas aeruginosa* ATCC 15442. All strains were obtained from the American Type Culture Collection 140 (Rockville, MD, USA) and were maintained in soft Tryptic Soy Agar (TSA) at 4°C. Initially, we screened the antibacterial activity of concentrated EO (4.35 mg/disc) in the agar disc diffusion assay according to the National Committee of Clinical Laboratory Standards protocol (NCCLS, 2003). For comparison, discs with sulfadiazine and cephalothin were used as standards. The minimum inhibitory concentration (MIC) of the EO (diluted in DMSO) for bacterials was determined against serial dilutions (0.97 to 500 µg/ml) using the broth microdilution method. The antifungal activity was evaluated against seven fungal strains obtained from the Department of Microbiology of the Federal University of

Pelotas. *Candida albicans*, *Candida guilliermondii*, *Candida globosa* and *Trichosporon asahii* were clinical isolates from sylvester animals; and *Candida parapsilosis*, *Candida lipolytica* and *Cryptococcus laurenti* were clinical isolates from bovine mastitic milk. Fungal strains were maintained on Potato Dextrose (PD) agar. Fungal cultures were subcultured (1% inoculum) in PD broth at 35°C for two to four days before screening. The MIC of EO was determined in concentrations ranging from 0.85 to 500 µg/ml (NCCLS, 2002). The MIC was recorded as the lowest EO concentration that inhibited fungal growth.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). For *in vitro* and *ex vivo* experiments, the data were validated using one-way analysis of variance followed by a Newman-Keuls test when appropriate. All *in vitro* tests were performed three times. The IC_{50} values (EO concentration providing 50% inhibition) were calculated from the graph of scavenging effect percentage *versus* EO concentration. Maximal inhibition (I_{max}) values were calculated at the most effective concentration were used. A *p* value less than 0.05 ($p < 0.05$) was considered significant.

Results

Chemical characterization of the EO

The EO was obtained in a 0.15% yield and is rich in sesquiterpenes and monoterpenes. According Table 1, the main components detected were linalool (**1**), fenchol (**2**), borneol (**3**), spathulenol (**4**) and globulol (**5**). The compounds 1,8-cineol (**6**), trans-linalool oxide (**7**) and *α*-terpineol (**8**) were also identified (Figure 1).

Table 1. Chemical composition of *Campomanesia xanthocarpa* leaves EO.

Peak[a]	Compound	RT[b]	Rel. %	KI (Lit)	Analysis
1	1,8-cineole	6.496	2.41		GC-MS
2	(*trans*)-linalool oxide	7.583	2.21		GC-MS
3	Linalool	8.343	34.92	1098 (1100)	GC-MS – KI
4	*α*-terpineol	8.785	1.79	1189 (1191)	GC-MS - KI
5	Spathulenol	10.37	7.84	1576 (1580)	GC-MS – KI
6	Fenchol	11.132	29.81	1117 (1115)	GC-MS - KI
7	Borneol	20.066	11.08	1165 - 1167	GC-MS – KI
8	Globulol	23.244	6.10	1583 - 1586	GC-MS - KI
Total			96.16%		

[a] Compounds listed in order of elution; [b] Retention time (in min.); KI= Kovats index (literature and experimental data).

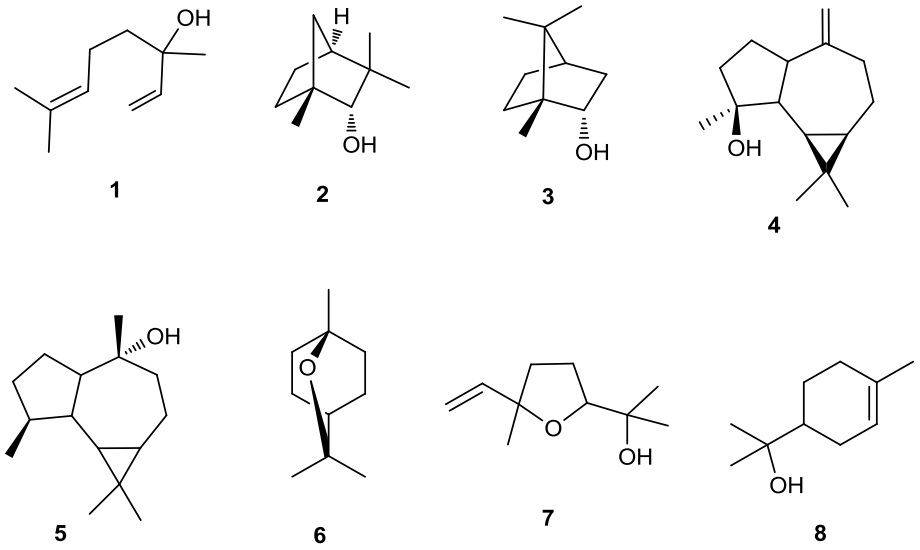

Figure 1. Major constituents and their percentage relative of EO from *C. xanthocarpa* leaves. linalool (**1**), fenchol (**2**), borneol (**3**), spathulenol (**4**), globulol (**5**), 1,8-cineole (**6**), (*trans*)-linalool-oxide (**7**), α-terpineol (**8**).

Antioxidant capacity

The antioxidant capacity *in vitro* of EO is presented in Table 2. The DPPH radical scavenging ability of EO was higher than that observed for stable $ABTS^{\bullet+}$

radicals, when IC_{50} values and evaluated concentration range were compared ($p < 0.05$).

The higher concentrations tested (1000 and 2000 µg/ml) of EO showed reducing capacity in FRAP assay. However, these concentrations could not be evaluated due the unsolubility in reaction medium of chelate ferrous ion assay. Just 100 µg/ml was effective to chelate ferrous ion.

In this study, the lipid peroxidation induced by different pro-oxidants of linoleic acid and of mice tissues (liver, kidney and brain regions) were measured by TBARS assay. The EO protected linoleic acid peroxidation induced by Fe^{2+}-ascorbic acid at concentrations ≥ 50 µg/ml (IC_{50} = 202.0 ± 5.4 µg/ml). Also, the EO (1000-2000 µg/ml) had significantly decreased the lipid peroxidation induced by Fe^{2+}-EDTA in all of analyzed tissues (liver, kidney and brain) at concentration 1000-2000 µg/ml. In this way, at a concentration of 2000 µg/ml of EO, the liver lipid peroxidation (48.65 ± 8.35 %) was equivalent to the control value (basal value 33.93 ± 5.28 %).

Our investigation of antioxidant capacity of EO against the lipid peroxidation induced by SNP was expanded to hippocampus, cortex and cerebellum. The antioxidant capacity was effective at concentrations greater than 25 µg/ml in brain regions and the higher potential was observed in cortex, followed by cerebellum and hippocampus, where IC_{50} values were comparable (Table 2).

Pro-oxidant capacity

The effect of the EO on the *in vitro* δ-ALA-D activity in mouse liver, kidney and brain was measured as a parameter of the pro-oxidant activity, once the enzymatic sulfhydryl groups can be inhibited in the presence of a pro-oxidant agent (Folmer et al., 2003). The measured δ-ALA-D activity demonstrated that the EO did not alter enzyme activity in the liver, kidney or brain homogenates at the tested concentrations (100-2000 µg/ml) (data not show).

Table 2. DPPH and $ABTS^{\bullet+}$ radical scavenging capacity, ferric ion reducing antioxidant power (FRAP), chelating activity of ferrous ion (Fe^{2+}) and lipid peroxidation induced by Fe^{2+}-ascorbic acid (linoleic acid), Fe^{2+}-EDTA (liver, kidney and brain) and SNP (hippocampus, cortex and cerebellum) inhibition of *Campomanesia xanthocarpa* leaves EO.

Assay	Effective concentrations (µg/ml)	IC_{50} (µg/ml)	I_{max} (%)
% DPPH scavenging activity	50-2000	1710.0 ± 5.3	57.6 ± 3.0
% $ABTS^{\bullet+}$ scavenging activity	500-2000	1980.0 ± 6.3	49.5 ± 10.0
FRAP	1000-2000	nd	-
Chelating activity ferrous ion	100	nd	12.66 ± 5.96
Lipid peroxidation:			
Linoleic acid	50-2000	202.0 ± 5.4	58.8 ± 9.0
Liver	2000	1960.0 ± 6.2	52.0 ± 4.0
Kidney	1000-2000	nd	21.0 ± 6.0
Brain	1000-2000	nd	29.5 ± 2.0
Hippocampus	25-100	64.5 ± 8.0	65.7 ± 13.0
Cortex	25-100	40.0 ± 6.2	72.0 ± 8.0
Cerebellum	25-100	52.0 ± 4.5	73.3 ± 9.0

Data of IC_{50} and I_{max} are expressed as mean ± SD. nd: not detected.

Antimicrobial activity

The antimicrobial activity of the EO was evaluated against seven bacterial strains using agar disc diffusion assay. The EO displayed a variable degree of antibacterial activity and had lower inhibition zone against the different strains tested when compared to standards antibiotics (sulphadiazine and cephalotine). As represented in Table 3, *S. Aureus, S. typhimurium*, *A. hydrophila* and *P. aeruginosa* had reduced growth in the presence of EO. In order of antibacterial effectiveness of EO, relative to MIC values, our results showed the effectivity order against *P. aeruginosa* > *S. typhimurium* > *S. aureus* = *A. hydrophila.* On the other hand, the EO

has not reduced the grownt of *L. monocytogenes*, *S. dysenteriae* and *E. coli* at the tested concentrations.

The EO presented inhibitory effect on the growth of all studied fungal strains with MIC values ranging from 125 to 250 µg/ml.

Ex vivo assay

A single oral dose (100 and 500 mg/kg) of EO did not cause mouse mortality and did not alter body weight gain. The cerebral, hepatic and renal TBARS and ascorbic acid levels were unchanged when compared to the control group (canola oil). Besides, treatment with the EO didn’t alter CAT and δ-ALA-D activities in all of analized tissues (data not shown).

Table 3. Antimicrobial activity of *Campomanesia xanthocarpa* leaves EO.

Bacterial strain	MIC (µg/ml) [e]	Inhibition zone (mm)[b]		
	EO	EO	Sulphadiazine	Cephalotine
Listeria monocytogenes	nd	8.6 ± 0.5	30	24
Staphylococcus aureus	125	20.6 ± 0.5 [c]	36	40
Salmonella typhimurium	62.5	11.3 ± 1.1 [d]	44	28
Shigella dysenteriae	nd	nd	30	26
Escherichia coli	nd	nd	28	32
Aeromonas hydrophila	125	13.6 ± 3.7 [c]	20	24
Pseudomonas aeruginosa	31.2	18.3 ± 1.5 [c]	22	27
Fungal strain	**MIC (µg/ml) [e]**			
Candida globosa	125			
C. guilliermondii	125			
C. lypolytica	125			
C. parapsilosis	125			
C. albicans	125			
Trichosporon asahii	125			
Cryptococcus laurentii	250			

The values were analyzed by one-way ANOVA, followed by the Newman–Keuls multiple comparison test; each value is expressed as the mean ± SD (n=3). [b] The concentrations of EO, sulphadiazine and cephalotine on disc were 4.35 mg/disc, 5.00 and 5.00 µg/disc, respectively. [c] denotes $p < 0.001$, [d] denotes $p < 0.01$ when compared with the respective diameter of the paper disc (6 mm); [e] MIC = minimal inhibitory concentration (µg/ml).

Discussion

This study evaluated the chemical composition, antioxidant and antimicrobial effects of *Campomanesia xanthocarpa* leaves EO. The chemical composition of EO used in this study is in partial agreement with those reported by other authors, which reported (*E*)-nerolidol, linalool, spathulenol, globulol and epi-globulol as major components (Limberger et al., 2001). Several factors alter the EO chemical composition, such as physiologic, climatic, environmental, geographic and genetics

conditions (Figueiredo et al., 2008). These reasons can explain the difference in EO profile composition among different species of *Campomanesia,* like *C. adamantium* (Cambess.) O. Berg leaves EO, rich in terpenes (Coutinho et al., 2009) and some similarities with *C. Rhombea* O. Berg leaves EO, that has linalool and globulol as the major constituents (Limberger et al., 2001).

The reducing power of the EO was supported by the DPPH radical scavenging assay, which, like FRAP assay, evaluate the ability of antioxidants to transfer a single electron to unstable species. These findings are consistent with antioxidant capacity of some oxygenated monoterpene-rich (mainly alcohols) EOs, such as *Cananga odorata* (Lam.) Hook.f. & Thomson (linalool), *Rosmarinus officinalis* L. (borneol and tepineol) and *Thymus vulgaris* L. (carvacrol and geraniol), which showed scavenging capacity of DPPH radicals ranging from 59.6 ± 0.42% to 75.6 ± 0.53% (Sachetti et al., 2005). EO was also capable to react with $ABTS^{\bullet+}$ radicals, which are more reactive than DPPH ones, and involve also a preferential electron-transfer process (Kaviarasan et al., 2007). On the other hand, this effect was oberved only at concentrations of 500-2000 µg/ml of EO, indicating a lower efficacy compared to its DPPH scavenging capacity.

The chelate effect of EO at 100 µg/ml, indicates indirect protection against $^{\bullet}OH$ radicals, which are produced by Fenton-type reactions involving H_2O_2 and transition metals, like ferrous ion (Fe^{2+}), which can oxidize lipids, proteins and DNA in live systems. When the complex Fe^{2+}-ascorbic acid was used as pro-oxidant agent in linoleic acid emulsion, the EO presented antioxidant capacity, which could be associate at least in part to its chelating property.

The lipid peroxidation inhibition is an important effect to be analised because it is involved in several chronical diseases. Interestingly, the present study showed that EO inhibited in vitro lipid peroxidation of mice tissues induced by different pro-oxidants. The ability of EO to decrease lipid peroxidation of liver, kidney and brain, induced by Fe^{2+}-EDTA complex, ranging to 1000-2000 µg/ml, can be associated with the capacity to scavenging free radicals, because when EDTA forms a complex with iron (II), hydroxyl radicals are generated in the system. Compared to the liver and other organs, the brain contains high levels of polyunsaturated fatty acids and relatively low levels of enzymatic and non-enzymatic antioxidants. Many authors have shown that the cortex and hippocampus are more susceptible to oxidative

damage as compared to the cerebellum and thus has an increased risk of neurodegenerative disorder (Mandavilli & Rao, 1996). According these considerations, we studied the antioxidant capacity of EO in the lipid peroxidantion of brain regions indued by SNP. SNP induces neurotoxicity by generating •OH and NO release, leading to oxidative stress and injury in brain tissue (Requena et al., 2003). Similarly to the antioxidant capacity of the EO observed in mice tissues in this study, the protective effect in brain regions may be related to the capacity of EO in scavenging free radicals as •OH, generated by SNP.

Our results show that the EO has antimicrobial activity against important disease- and infection-causing strains. Antibacterial effect was not Gram-specific. This lack of Gram-specificity was also observed for other EOs, such as those of *Eucalyptus* species (*E. dives* Shauer, *E. olida* L.A.S. Johnson & K.D. Hil and *E. staigeriana* F. Muell. ex F.M. Bailey) (Gilles et al., 2010) and *Artemisia annua* L. (Cavar et al., 2012). The EO from *Tarchonanthus camphoratus* L. leaves, rich in fenchol **2** (15.9%), 1,8-cineole **6** (14.3%) and *α*-terpineol **8** (13.2%) also showed antimicrobial effect against *S. aureus* and *P. aeruginosa* (Matasyoh et al., 2007).
According our data, C. xanthocarpa EO had a significant antibacterial effect against bacterial known for causing foodborne diseases, like *S. aureus*, *S. typhimurium*, *A. Hydrophila* and *P. aeruginosa,* which is frequently resistant to antibiotics (Janda & Abbott, 2010) and one of the bacterial responsible to biofilm development in food industry (Boyd & Chakrabarty, 1995). Additionally, EO was most efficient against *P. aeruginosa* than another bacterial strains. Moreover, *S. aureus* has been recognized worldwide as the etiological agent of most of foodborne diseases (Soares et al., 2011).

Several mechanism have been generally atributed to the antimicrobial effect of essential oils. The capacity of EO and isolated components to interact with membrane structures, by their lipophilic character, can lead to saturation, increase of membrane fluidity and dysfunction of membrane molecular structures (Sikkema et al.,1994; Turina et al., 2006).

In this context, it is difficult to attribute the activity of a complex mixture to a single constituent of EO. However, the antibacterial activity of EO present in this study, could be most likely due to the presence of linalool (**1**, Figure 1), one of the major component of *C. xanthocarpa* EO. Linalool is known to have the inhibitory

activtiy against several bacterial strains (Bagamboula et al., 2004; Hussain et al., 2008; Randrianarivelo et al., 2009; Duman et al., 2010; Ebrahimabadi et al., 2010). Fenchol (**2**, Figure 1) also showed significant antimicrobial activity against several bacterial strains, among which *P. aeruginosa* (Kotan & Kordoli, 2007). Thus, the antibacterial effect of EO of *C. xanthocarpa* may be attributed, at least in part, to the synergistic effect of those and the others constituents.

Some of fungal strains chosen in our study, like *C. albicans* and *C. parapsilosis,* are the main fungus responsible for invasive bloodstream infections and its cause mortality in immunocompromised patients (Selvarangan et al., 2003). *Trichosporon asahii* is the main etiologic agent of trichosporonosis in humans (Colombo et al., 2011) and *Cryptococcus laurentii* already has reported to be involved in meningitis (Kordossis et al., 1998). The EO of *C. xanthocarpa* leaves had a significant activity against these strains and Candida spp, which can suggest the potential to clinical aplication to prevent or delay fungal infections.

The general antifungal mechanism of action of some EOs has been related to lesions in the cytoplasmic membrane of strains. More specifically, isolated compounds from EOs, like carvacrol and thymol, have the ability to disrupt vesicles and cell membranes and to impair the ergosterol biosynthesis (an essential component of fungal plasma membrane) in *Candida* strains (Ghannoum & Rice, 1999; Ahmad et al, 2011). However, the mechanism of action of EO of *C. xanthocarp*a leaves and the most of its constituents against fungal studied here, are not weel documented and still unknown. Therefore, the antifungal activity of EO could be atributed, at least in part to the major and minor constituents, linalool and *α*-terpineol, respectively, which have antifungal activity against *Candida albicans* (MIC = 125 µg/ml and 500 µg/ml, repectively) (Ebrahimabadi et al. 2010).

Conclusions

The present study demonstrates the *in vitro* antioxidant capacity, mainly by inhibition of lipid peroxidation of EO of *C. xanthocarpa* leaves. The EO is also an effective inhibitor of bacterial and fungal growth. These findings may be attributed to the major compounds present in the EO. Furthermore, EO does not cause symptoms of toxicity in mice after oral administration. These findings suggest that *C.*

xanthocarpa leaves EO could be used as a natural antioxidant and antimicrobial agent.

Declaration of interest

The authors report no declarations of interest.

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5 Pesquisa exploratória sobre a capacidade antioxidante *in vitro* de araçá (*Psidium cattleianum*) e butiá (*Butia odorata*) liofilizados

A partir da revisão da literatura sobre as frutas nativas araçá e butiá, observou-se que ainda há poucos dados referentes à sua capacidade antioxidante. Portanto, neste trabalho foram utilizados diferentes métodos para a avialiação da capacidade antioxidante *in vitro* das frutas liofilizadas. O manuscrito científico foi submetido ao periódico *Food Chemistry*, ISSN: 0308-8146.

Antioxidant properties of fruits of *Psidium cattleianum* and *Butia odorata* grown in southern Brazil

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Abstract

Psidium cattleianum and *Butia odorata* plant species native from southern Brazil are known by their fruits: strawberry guava/araçá and butiá, respectively. In this study two variety of *B. odorata* (red and yellow) and one of *P. cattleianum* (red variety) were studied for their *in vitro* antioxidant properties. Red butiá possess higher concentrations of phenolic compounds, carotenoids, anthocyanins and ascorbic acid than yellow *B. odorata* and *P. cattleianum*. The lyophilized fruits showed antioxidant capacity against free radicals (DPPH, ABTS) and potential reduction power (FRAP). Different to the two varieties of *B. odoratta*, *P. cattleianum* was able to scavenge $OH^{\bullet}$. A high antioxidant effect (75.6 - 91.7%) was observed on lipid peroxidation of brain regions (hippocampus, cortex and cerebellum) of mice by all of fruits. Additional studies to identify the major bioactive compounds and *in vivo* effects of fruits must be developed for nutritional and therapeutic applications.
Key words: lipid peroxidation, free radicals, antioxidant, *P. cattleianum*, *B. odorata*.

1. Introduction

There are some evidences that the intake of fruits and vegetables are playing a beneficial role in the prevention and treatment of different chronic diseases, like cancer and neurodegenerative disorders (Rahman, 2007; Lobo, Phatak, & Chandra, 2010; Lü, Lin, Yao, & Chen, 2010; Singh, Chandra, Mahdi, Ray, & Sharma, 2010). The potential of beneficial plants has been attributed to the presence of bioactive compounds, such as flavonoids, anthocyanins, tannins and other polyphenolic compounds that show antioxidant properties (Crozier, Jaganath, & Clifford, 2009). Therefore, much attention has been focused on the use of natural antioxidants to protect the damage caused by free radicals. In recent years, the investigation of natural antioxidant sources has become important because they are safer and, in most cases, more efficient than synthetic compounds (Viuda-Martos, Navajas, Zapata, Fernandez-Lopez, & Perez-Alvarez, 2010).

In this context, the interest in edible tropical fruits has been increasing in developed countries due to their potential health benefits (Clerici, & Carvalho-Silva,

2011; Oliveira, Yamada, Fagg, & Brandão, 2012). *Psidium* genus (*Myrtaceae* family) includes *Psidium cattleianum* Afzel ex. Sabine and *Psidium guajava* L.*,* native plants whose fruits have nutritional and functional properties (De Souza, Haas, Poser, Schapoval, & Elisabetsky, 2003; Galho, Lopes, Bacarin & Lima, 2007; Gutiérrez, Mitchell, & Solis, 2008). Chemical composition and preliminary evaluations of pharmacological properties of *P. cattleianum* extracts have been described, as i*n vitro* antioxidant, antimicrobial and antiproliferative effects are atributed to acetone and aqueous extracts of two berries (red and yellow); and anti-inflammatory property is atributed to organic extracts and isolated compounds (Fetter, Vizzotto, Corbelini, & Gonzalez, 2010; Medina et al., 2011; McCook-Russell, Nair, Facey, & Bowen-Forbes, 2012)

Butia is a small genus of subtropical palms that occur in some countries of South America (Henderson, Galeano, & Bernal, 1995). Six species are known and found in Southern of Brazil (Rossato, Barbieri, Schäfer, & Zacaria, 2007), which *Butia odorata* and *Butia capitata* fruits are known as butiá or jelly palm fruit. Butiá fruits have a color that ranges from pale yellow to a bright reddish orange, strongly aromatic pulp, which is widely appreciated for preparing juices, jams and liquor (Rosa, Castellani, & Reis, 1998). The nutritional importance of *Butia* species as *B. capitata* fruits is linked to content of carotenoids, provitamin A and fatty acids (Lopes et al., 2012). Previous data have reported the *in vitro* free radicals scavenging and antidiabetic capacity of *B. capitata* fruits (Gonçalves, Lajolo, & Genovese, 2010; Pereira et al. 2013).

To the best of our knowledge, there are few studies regarding the biological properties related to the antioxidant capacity of *B. odorata* and *P. Cattleyanum.* Therefore*,* this study aimed to evaluate the phytochemical composition and *in vitro* antioxidant properties of *B. odorata* and *P. Cattleianum* fruits.

2. Materials and methods

2.1.Plant materia,l animals and reagents

P. cattleyanum fruits were obtained from Embrapa Clima Temperado (Pelotas/RS/Brazil), and *B. odorata* fruits (red and yellow varieties) were collected in

the rural area of the municipality of Tapes (RS/Brazil) between February-March, 2012. Fruits free of defects were selected, whashed and the pulp (along with peel) was manually separated from the seeds, using a stainless knife and than was lyophilized. Samples were storage at -80 °C until analysis.

For the antioxidant assays, the samples were diluted (1-500 µg/mL) in dimethyl sulfoxide (DMSO) and centrifuged at 2000 g for 10 min to separate the insoluble fibers of fruits. The supernatant was used in the antioxidant assays.

The reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radical cation were supplied by Sigma (St. Louis, USA); sodium nitroprusside and ferric chloride (Vetec, Brazil). All other reagents and auxiliaries were of analytical grade.

Healthy male Swiss albino mice (total 8 animals), with an average body weight 30 ± 5 g and were obtained from the Central Biotherium of Federal University of Pelotas (Brazil). The Committee on Care and Use of Experimental Animal Resources, the Federal University of Pelotas, Brazil approved the experimental protocols for this study.

2.2. Chemical composition of samples

2.2.1. Phenolic compounds content

Total phenolic content was determined using the method described by Singleton and Rossi (1965). Methanol (20 mL) was added to a sample of lyophilized fruits (2.0 g). The mixture was homogenized every 5 min for 3 h at room temperature (20 ± 3 °C), and after that it was filtered through cotton. The homogenate was transfered to a 50 mL volumetric flask, completing the volume with methanol. To accomplish the quantification of total phenolic compounds, it was used 1 mL of filtered extract, to which 1.5 mL of 20% of sodium carbonate solution in 0.1 M NaOH was added. The mixture was left 2 h in a water bath at 37 °C and then it was added 0.5 mL of Folin-Ciocalteau diluted (1:2, v/v) in ultrapure water. The reading was done in a spectrophotometer (Ultrospec 2000 model) at 765 nm using methanol as blank. It was also prepared the standard curve of gallic acid for the quantification of phenolic compounds. The results were expressed in mg of galic acid per 100 g of sample.

2.2.2. Anthocyanins content

Total anthocyanin content was determined using 1 g of sample that was suspended in 25 mL of cold ethanol containing 0.01% v/v HCl. The mixture was left for 1 h in the dark, shaking every 5 min. The mixture was filtered and transferred to a volumetric flask with total volume (50 mL) completed with ethanol acidified. The anthocyanin content was measured at 520 nm in a spectrophotometer, and total anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalents per 100 g of sample (Lees, & Francis, 1972).

2.2.3. Carotenes content

Frozen sample, equivalent to 5 g, and 2 g of celite® were ground under liquid nitrogen using a mortar and pestle, suspended in 20 mL of acetone (80% v/v), stirred for 10 min and filtered (the extraction was repeated three times). The filtrate was then transfered to a separatory funnel and 30 mL of petroleum ether and 100 mL of water were added. The upper ethereal layer was transfered to a 50 mL volumetric flask and the volume completed with petroleum ether. Absorbance was measured at 470 nm in an UV/Vis spectrophotometer. Total carotene content was determined using the equations described by Rodrigues-Amaya (2001), and expressed as mg of β-carotene per 100 g of sample.

2.2.4. L-ascorbic acid

L-ascorbic acid was determined using the method described by Vinci, Rot and Mele (1995) and by Ayhan, Yeom and Zhang (2001). The sample (10 g) was ground using a mortar and pestle, suspended in 30 mL of a cold metaphosphoric acid solution (4.5% w/v in water, 4 °C), stored at 4 °C for 1 h in the dark and then the volume was brought to 50 mL with distilled water. The mixture was filtered and the filtrate centrifuged at 12.000 g for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm Durapore membrane, and a 25 µL aliquot was injected in a HPLC Shimadzu system, using a reverse phase Shimadzu (Kyoto, Japan) Shim-Pak CLC-

ODS column (3.9 cm x 150 mm x 4 µm). An elution gradient started at 100% acetic acid 0.1% v/v (A), then linearly reduced to 98% of A and 2% of methanol (B) at 5 min; then held for 2 min and returned to initial conditions at 10 min. Flow rate was 0.8 $mL.min^{-1}$ and the UV detector was set at 254 nm. Identification was based on retention time comparison to an *L*-ascorbic acid standard (Synth, Diadema, SP, Brazil). Quantification was based on an external standard calibration curve and results were expressed as mg of *L*-ascorbic acid per 100 g of sample.

2.3. Antioxidant activity

2.3.1. Diphenyl-2-picryl- hydrazyl (DPPH) free radical-scavenging assay

Measurement of the scavenging activity of the fruits against the DPPH radicals was performed in accordance with method reported by Choi et al. (2002). Briefly, 50 µM of $DPPH^{\bullet}$ (990 µL) in methanol was added to medium containing different concentrations of samples (5-500 µg/mL) and incubated for 30 min at 30 °C. The absorbance of the resulting solutions was recorded spectrophotometrically at 517 nm. A decrease in absorbance indicates DPPH free radical scavenging activity. The percentage of scavenging DPPH radical was calculated from equation:

$$DPPH^{\bullet}\ scavenging\ (\%) = (A_c - A_s / A_c) \times 100$$

where A_c is the absorbance of the control reaction mixture (excluding fruits samples) and A_s is the absorbance of the reaction mixture with different concentrations of fruits.

2.3.2. 2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$) scavenging activity

$ABTS^{\bullet+}$ scavenging activity of fruits was evaluate according method described by Re et al. (1999). In this assay the oxidation of ABTS aqueous solution (2 mM) was carried by addition of potassium persulfate (2.45 mM). The reaction mixture is stored in the dark at room temperature for 12-16 hours. Before usage, the $ABTS^{\cdot+}$ solution was diluted to give an absorbance value of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, $ABTS^{\cdot+}$ solution was added to the fruit at

different concentrations (10-500 µg/mL) and placed for 30 min at room temperature. A decreasing in the absorbance of the samples indicates $ABTS^{\bullet+}$ radical scavenging activity. The percentage of scavenging $ABTS^{\bullet+}$ was calculated from equation:

$$ABTS^{\bullet+} \text{ scavenging (\%)} = (A_c - A_s/A_c) \times 100$$

where A_c is the absorbance of the control sample ($ABTS^{\bullet+}$ solution excluding the fruit sample) and A_s is the absorbance of the reaction mixture with fruits at different concentrations.

2.3.3. Hydroxyl radical ($^{\bullet}OH$) scavenging activity

Hydroxyl radical-scavenging activity of fruits was evaluated according to the method described by Smirnoff and Cumbes (1989), with some modifications. The assay involves the reaction of sodium salicylate (20 mM), $FeSO_4$ (1.5 mM) with the fruit samples at different concentrations (10-500 µg/mL). The reaction started with the addition of H_2O_2 (6 mM). The reaction mixture was allowed in water bath at 37 °C for 1 h. The absorbance of the samples was recorded at 510 nm and the radical-scavenging activity was calculated from equation:

$$^{\bullet}OH \text{ scavenging (\%)} = (A_c - A_s/A_c) \times 100$$

where A_c is the absorbance of the control sample (reaction mixture in the absence of tested fruit sample) and A_s is the absorbance of the reaction mixture with fruit sample in different concentrations.

2.3.4. Ferric ion reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was measured according to the method described by Stratil, Klejdus and Kuban (2006) with slight modifications. The FRAP reagent was prepared by mixing aqueous solutions of sodium acetate buffer (38 mM, pH 3.6), $FeCl_3 \cdot 6H_2O$ (20 mM) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (10 mM in 40 mM of HCl) in a 10:1:1 ratio. This reagent was freshly prepared before each experiment. For each assay, 10 µL of fruit sample at different concentrations (1-250 µg/mL) and 990 µL of the FRAP reagent were added. The reaction mixture was incubated in the dark at 37 ºC for 40 min. Control was a sample with 10 µL of DMSO and 990 µL of the FRAP reagent. The absorbance of the resulting solution was

recorded at 593 nm and results were expressed in absorbance values and compared to control sample.

2.4. Lipid peroxidation on brain structures

Swiss male mice were euthanized by cervical dislocation, and the brain was rapidly removed and placed on ice. The cortex, hippocampus and cerebellum were dissected and kept chilled and homogenized in 50 mM Tris-HCl at pH 7.4 (1/5 w/v). The samples were centrifuged for 10 min at 2400 g. The pellet was discarded and a low-speed supernatant from each tissue was used for lipid peroxidation assay.

To induce lipid peroxidation, an aliquot of each tissue sample (20 µL for cortex and cerebellum; and 10 µL for hippocampus) was incubated for 1 h at 37 °C with 10 µL of fruits sample (5 - 500 µg/mL) and the pro-oxidant agent, sodium nitroprusside (SNP, 100 µM). The lipid peroxidation level was detected by TBARS assay, according the method reported by Ohkawa, Ohishi and Yagi (1979). A sample without SNP was used as control not induced. The results are expressed as percentage of lipid peroxidation and compared to induced sample (tissue + SNP = 100% of lipid peroxidation).

2.5. Statistical analysis

The results are expressed as the mean ± standard deviation (SD) and the data were validated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test, when appropriate. All tests were performed three times. The IC_{50} values (fruits concentration providing 50% effect) were calculated from the graph of effect percentage *versus* fruits concentration; maximal inhibition (I_{max}) values were calculated at the most effective concentration used. A *p* value less than 0.05 ($p < 0.05$) was considered significant.

3. Results and discussion

3.1. Chemical composition

The amounts of total bioactive compounds were expressed in relation to the dry weight of samples. The total phenolics, anthocyanins, carotenoids and ascorbic acid content of three fruits are show in Table 1. Red *B. odorata* fruit had more total phenolic compounds than *P. cattleianum* ($p < 0.001$) and yellow *B. odorata* ($p < 0.001$) fruits. Red *B. odorata* fruit also showed a significant difference of carotenoids, ascorbic acid and anthocyanins concentration of two other tested fruits. Total carotenoids of red butiá was approximately 3 and 8 times higher than yellow *B. odorata* fruit ($p < 0.001$) and red *P. cattleianum* fruit ($p < 0.001$) content, respectively. Additionally, ascorbic acid content of red butiá was 2.5 times higher than in yellow butiá ($p < 0.001$) while anthocyanin content was 12 % and 24.5 % higher than observed in yellow butiá ($p < 0.001$) and in *P. cattleianum* fruits ($p < 0.01$), respectively.

Carotenoids content in yellow butiá was 2.5 times higher than in *P. cattleianum* ($p < 0.001$). On the other hand, *P. cattleianum* contains around 21 % more anthocyanins than yellow butiá ($p < 0.01$).

Previous studies showed the phenolic compounds content (163-259 mg catechin/100g fresh pulp) and ascorbic acid (53 mg/100g fresh pulp) of *B. capitata* fruits (Faria, Almeida, Da Silva, Vieira, & Agostini-Costa, 2008). Faria et al. (2011) described the carotenoids profile of *B. capitata* fruits (ranging from 11.1 to 43.9 µg/g fresh fruit) and suggested that the fruits are a good dietary source of β-carotene and provitamin A. More recently, Pereira et al. (2013) also showed high carotenoids (39.77 µg/g fresh fruit), Vitamin C (320 µg/g fresh fruit) and phenolic content (636.95 mg chlorogenic acid equivalents/100 g fresh fruit) of *B. capitata* fruits (pindo palm fruit) cultivated in southern Brazil. Our results (Table 1) show highest content of carotenoids, phenolic compounds and ascorbic acid when compared to other studies, it can be explained by the use of low moisture samples (dried fruits instead fresh *B. odorata* fruits) and different regions of collection (Southeast and South Brazil).

Table 1 - Bioactive compounds of *P. Cattleianum* and two varieties of *B. odorata* lyophilized fruits.

Sample	Phenolic compounds [(A)]	Anthocyanins [(B)]	Carotenoids [(C)]	Ascorbic Acid [(D)]
P. cattleianum	875.43 ± 7.44 [a]	143.17 ± 4.76 [a]	29.63 ± 1.75 [a]	16.31 ± 0.12 [a]
Yellow *B. odorata*	864.42 ± 7.13 [a]	126.25 ± 6.69 [b]	74.09 ± 1.39 [b]	77.45 ± 4.08 [b]
Red *B. odorata*	978.39 ± 12.91 [b]	171.00 ± 5.06 [c]	234.04 ± 5.19 [c]	197.99 ± 4.37 [c]

[(A)] mg galic acid/100g sample; [(B)] mg cyanidin-3-glucoside/100g sample; [(C)] µg lycopene/100g sample; [(B)] mg ascorbic acid/100g sample. Values expressed in dry weight (d.w.) of samples. Means followed by different letter in the same column are significantly different by Student-Newman-Keuls test for post-hoc comparison.

Previous data also showed that *P. cattleianum* fruits are rich in bioactive compounds. Fetter et al. (2010) showed the phenolic compounds (668.63 ± 41.32 mg chlorogenic acid/100g fresh fruit), carotenoids (1.07 ± 0.08 mg β-carotene/100 g fresh fruit) and anthocyanin contents (36.12 mg/100 g fresh fruit) of *P. cattleianum*. In this line, Medina et al. (2011) also found a high phenolic concentration in water and acetone extracts of *P. cattleianum* (655.36-657.97 mg of GAE/100 g fresh fruit) cultivated in southern Brazil and when compared to yellow *P. cattleianum*, phenolic content of red of *P. cattleianum* was higher than of yellow of *P. cattleianum*. Our results showed difference in total bioactive compounds analyzed when compared to the previous studies, cause are expressed in fruits dried weight.

3.2. Radical scavenging activity

The results of DPPH, ABTS and OH radicals scavenging capacity of *B. odorata* and *P. cattleianum* are presented in Table 2. The DPPH and ABTS radicals scavenging capacity of *P. cattleianum* presented significant effect at lower concentration (10 µg/mL) than red and yellow butiá (50 µg/mL). Additionally, the potential antioxidant effect of *P. cattleianum* against DPPH radicals was confirmed when compared IC_{50} values of three fruits, decreasing in the order of *P. cattleianum* >

red *B. odorata* = yellow *B. odorata* ($p < 0.01$). On the other hand, no difference was observed between fruits against ABTS radicals (Table 2). To scavenge ABTS radicals, which are more reactive than DPPH radicals, electron transfer reactions are usual. To neutralize DPPH radical, the antioxidants have H atom transfer capacity (Kaviarasan, Naik, Gangabhagirathi, Anuradha, & Priyadarsiniln, 2007). As described by McCook-Russell et al. (2012), the methanolic extract of *P. cattleyanum* fruits, rich in phenolic compounds and ascorbic acid, has highest antioxidant potential in ABTS assay, but the isolated compounds (triolein, *β*-sitosterol, ursolic acid, oleanolic acid, 2*α*-hydroxyursolic acid, 2*α*-hydroxyoleanolic acid and citric acid) did not have antioxidant activity. Luximon-Ramma et al. (2003) also showed the antioxidant capacity of red *P. cattleianum*, which was higher than others Mauritian exotic fruits on ABTS assay.

Only *P. cattleianum* presented OH radical scavenging capacity (IC_{50} = 319.0 ± 2.0 µg/mL and I_{max} = 67.3 ± 5.6%). The total antioxidant properties can be associated to specific compounds or a synergic effect from major and minor compounds present in red *P. cattleianum* fruits, like (-)-epicatechin, gallic acid, coumaric acid, ferulic acid, myricetin and quercetin (Medina et al., 2011).

Most of works have showed no correlation between antioxidant capacity and total anthocyanin and carotenoids contents (Fetter et al., 2010) and a strong correlation with the amount of phenolic compounds and ascorbic acid in fruits (Luximon-Ramma et al., 2003).

Table 2 - DPPH, ABTS and OH radical scavenging capacity of *P. cattleianum* and *B. odorata* fruits.

Sample	DPPH		ABTS		•OH	
	IC_{50} (µg/mL)	I_{max} (%)	IC_{50} (µg/mL)	I_{max} (%)	IC_{50} (µg/mL)	I_{max} (%)
P. cattleianum	25.0 ± 10.00[a]	97.54 ± 4.20[a]	38.33 ± 9.20[a]	94.19 ± 9.2[a]	319.00 ± 2.0	67.30 ± 5.50
Yellow *B. odorata*	98.3 ± 2.02[b]	89.31 ± 6.38[a]	44.00 ± 5.30[a]	96.55 ± 1.17[a]	ns	ns
Red *B. odorata*	108.7 ± 33.77[b]	93.62 ± 2.20[a]	38.83 ± 6.00[a]	92.43 ± 9.54[a]	ns	ns

Data are presented as the mean ± S.D. $I_{max:}$ Maximal inhibition. IC_{50}: concentration required for 50% scavenging free radicals (DPPH, ABTS and OH); Values followed by different letter in a column are significantly different by Student-Newman-Keuls test for post-hoc comparison ($p < 0.01$). ns: no significant.

In addition, *P. cattleianum* (yellow and red) methanolic and aqueous extracts had antioxidant capacity on DPPH assay and protective effect against oxidative stress induced by H_2O_2 in eukaryotic cells (*Saccharomyces cerevisiae*) (Medina et al., 2011). Our results showed that the individual reaction of *P. cattleianum* on $^{\bullet}OH$ scavenging assay is one of the mechanism of action antioxidant that differ from butiá fruits. In this way, recently Ribeiro et al. (2014) showed the ability of *P. cattleianum* ethanolic extract (from skin and pulp of fruit) to scanvenge several reactive species, such as nitric oxide ($^{\bullet}NO$), peroxynitrite ($ONOO^-$), H_2O_2, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet}$) and hypoclorous acid (HOCl). This antioxidant capacity was atributed mainly to pulp extract, which has more phenolic compounds like ellagic acid and epicatechin (Ribeiro et al., 2014).

A powerful antioxidant often acts as a potent reductant agent. The FRAP assay measures the ability of an antioxidant reduce Fe(III)-triazine complex to Fe(II)-triazine complex. As shown in Table 3, significant FRAP's potential were observed and the reducing power scale was red *B. odorata* = *P. cattleianum* > yellow *B. odorata*.

Table 3 - Ferric reducing-antioxidant power (FRAP) of red *P. cattleianum*, yellow and red *B. odorata*.

Concentration (µg/mL)	Red araçá	Yellow *B. odorata*	Red *B. odorata*
1	0.179 ± 0.00 [a]	0.206 ± 0.03	0.413 ± 0.138[a]
10	0.307 ± 0.02[c]	0.327 ± 0.09[b]	0.442 ± 0.104[a]
50	0.877 ± 0.01[c]	0.549 ± 0.121[b]	0.670 ± 0.09[c]
100	1.489 ± 0.02[c]	0.875 ± 0.124[b]	0.962 ± 0.08[c]
250	max	1.678 ± 0.141[c]	1.829 ± 0.08[c]

Data are presented as the mean ± S.D. of absorbance at 593 nm. Values followed by different letter in the same column are significantly different of control sample (FRAP solution) by Student-Newman-Keuls test for post-hoc comparison; (a) $p < 0.05$; (b) $p < 0.01$ and (c) $p < 0.001$. (max) = maximal absorbance detected.

3.2. SNP-induced lipid peroxidation assay on brain regions

The brain tissue has many factors that lead to highest susceptibility to oxidative damage: polyunsaturated fatty acids content, fast oxidative metabolism (consume of 20% of cardiac output) and low levels of enzymatic and non-enzymatic antioxidants when compared to other organs. Oxidative damage in cortex and hippocampus can increase risk of neurodegenerative disorder, which needs to alternatives to improve the antioxidant status in the brain. In addition to screening their antioxidant properties, all tested fruits were evaluated regarding the protection against lipid peroxidation on hippocampus, cortex and cerebellum. As it can be seem in Figures 1-3, the three fruits were remarkably active in all studied brain tissues.

The fruits red araçá (IC_{50} = 17.5 ± 11.0 µg/mL) and red butiá (IC_{50} = 31.3 ± 7.0 µg/mL) presented antioxidant potential on hippocampus lipid peroxidation from 5 µg/mL, and yellow butiá (IC_{50} = 28.0 ± 3.4 µg/mL) shown significant effect from 50 µg/mL (Figure 1). When comparing IC_{50} and I_{max} values, araçá had the higher antioxidant potential on hippocampus. In addition, at concentration of 50 µg/mL, red araçá and red butiá decreased lipid peroxidation in hippocampus to values lower than the control sample (without SNP).

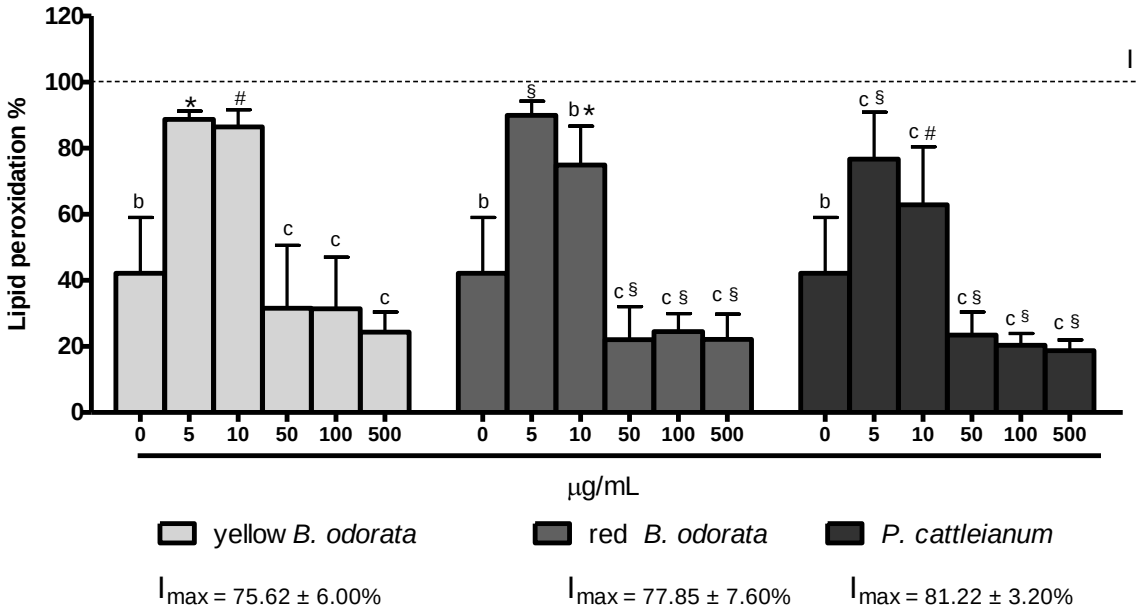

Figure 1 - Effect of yellow and red *B. odorata*, *P. cattleianum* on SNP-induced lipid peroxidation on hippocampus of mice. Data are presented as the mean ± S.D. (n = 3). The values are expressed as a % of lipid peroxidation and were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Small letters represent significant difference from the induced sample (I) (^{b} $p < 0.01$ and ^{c} $p < 0.001$), the symbols represent significant difference from the control sample (0) ($^{\#}p < 0.05$, * $p < 0.01$ and § $p < 0.001$.

Cortex lipid peroxidation was inhibited by red araçá (IC_{50} = 8.0 ± 2.50 µg/mL), red butiá (IC_{50} = 34.0 ± 9.23 µg/mL) from 5 µg/mL and yellow butiá (IC_{50} = 9.33 ± 7.50 µg/mL) from 50 µg/mL (Figure 2) and shown a maximal inhibition effect (I_{max}) ranging from 87.8 to 91.7%. If the IC_{50} values are compared, red araçá and yellow butiá had the same antioxidant potential and these were higher than that of red butiá ($p < 0.001$).

Cerebellum lipid peroxidation was decreased by *P. cattleianum* (IC_{50} = 44.67 ± 23.86 µg/mL), yellow butiá (IC_{50} = 32.67 ± 9.45 µg/mL) and red butiá (IC_{50} = 30.67 ± 5.00 µg/mL) (Figure 3). No difference among fruits was observed when IC_{50} values on cerebellum were compared.

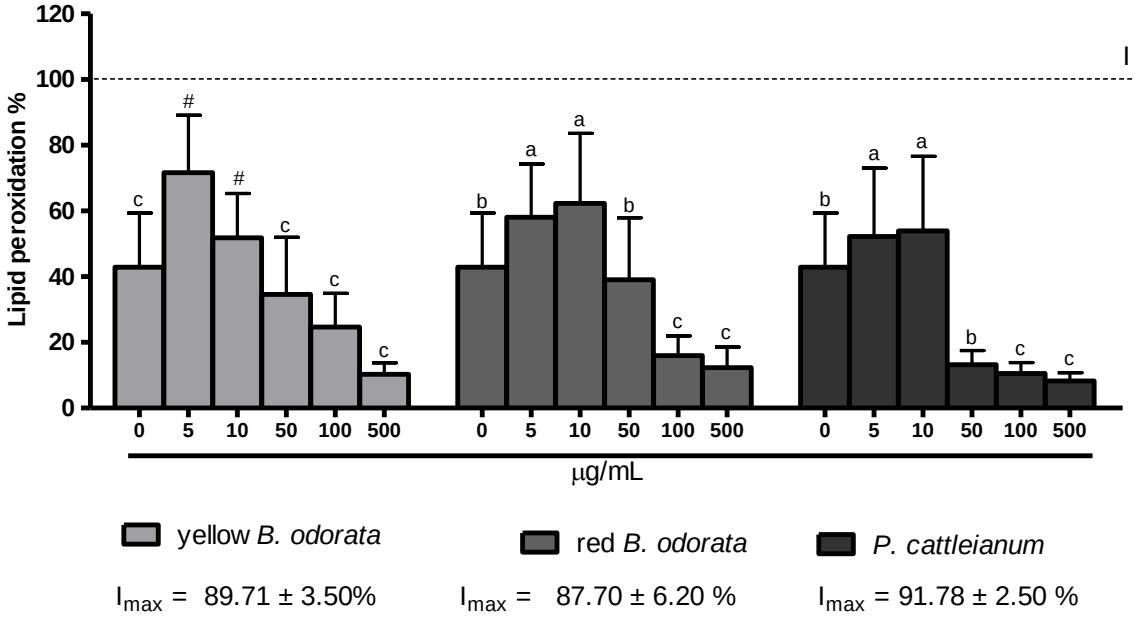

Figure 2: Effect of yellow and red *B. odorata*, *P. cattleianum* on SNP-induced lipid peroxidation on cortex of mice. Data are presented as the mean ± S.D. (n = 3). The values are expressed as a % of lipid peroxidation and were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Small letters represent significant difference from the induced sample (I) ($^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$), the symbols represent significant difference from the control sample (0) ($^{\#}p < 0.05$, * p <0.01 and $^{\S}p < 0.001$.

Taken together, all fruits studied had a potential antioxidant property in all brain structures, ranging from 75.6 to 91.7% of inhibition of SNP-induced lipid peroxidation.

Some of bioactive compounds present in studied fruits, like phenolic compounds, have powerful antioxidant properties, as can be seen by *in vitro* assays, such as inhibition of lipid peroxidation by scavenging hydroxyl radical ($^{\bullet}OH$), nitric dioxide (NO_2) and peroxynitrite acid (ONOOH) (Rice-Evans, Miller, & Paganga, 1996). Also, the antioxidant ability of fruits preventing the lipid peroxidation can be attributed to its carotenoids content, which react with peroxyl radicals before they propagate the lipid oxidation (Palace, Khaper, Qin, & Singal, 1999).

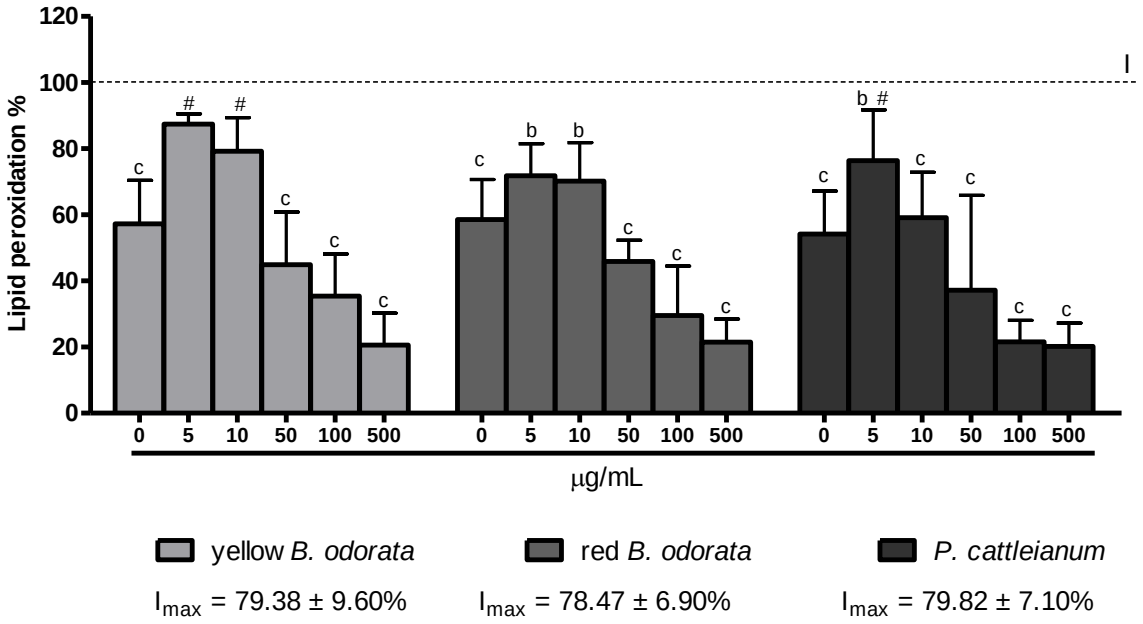

Figure 3: Effect of yellow and red *B. odorata*, *P. cattleianum* on SNP-induced lipid peroxidation on cerebellum of mice. Data are presented as the mean ± S.D. (n = 3). The values are expressed as a % of lipid peroxidation and were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Small letters represent significant difference from the induced sample (I) (^{a} $p < 0.05$, ^{b} $p < 0.01$ and ^{c} $p < 0.001$); the symbols represent significant difference from the control sample (0) ($^{\#}p < 0.05$, * $p < 0.01$ and § $p < 0.001$).

Several experimental studies have been demonstrated that fresh/dried fruits and their extracts can protect tissues against biomolecules oxidation. In this line, frozen açaí (*Euterpe oleraceae* Mart.) pulp had decreasing effect on lipid peroxidation of cortex, hippocampus and cerebellum of rats when induced by H_2O_2 (Spada, Dani, Bortolini, Funchal, Henriques & Salvador, 2009). Aqueous and ethanolic extracts from dried *Citrus unshiu* peel showed the capacity to decrease lipid peroxidation induced by Fe^{2+}/ascorbate on rat brain, which was atribute in part to the catechins and anthocyianidins content (Kim, 2013). Ethanolic extract from *Zanthoxylum alatum* fruits had an antioxidant effect on brain, liver and kidney of rats against Fe^{2+} and SNP induced lipid peroxidation that were related to the phenolic and flavonoid content (Batool, Sabir, Rocha, Shah, Saify & Ahmed, 2010).

According our results, *P. cattleianum* and *B. odorata* fruits can also protect the damage induced by a pro-oxidant agent on brain tissue, probably by interaction with intermediates on lipid peroxidation process like lipid peroxides.

4. Conclusions

The results of the present study show the significant antioxidant capacity of fruits in chemical and biochemical *in vitro* assays. All studied fruits are sources of remarkably antioxidant compounds and their antioxidant potential was significant by interacting with free radicals (DPPH and ABTS), potential reduction power (FRAP assay). Particularly, *P. cattleianum* was able to scavenge hydroxyl radicals. The fruits also had protective effect against lipid peroxidation in the brain structures of mice and *P. cattleianum* had the highest antioxidant potential. Additional studies to identify the main bioactive compounds present in those fruits and possibly antioxidant effects *in vivo* may be further developed for their nutritional and therapeutic applications.

Acknowledgements

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Viuda-Martos, M., Navajas, Y.R., Zapata, E.S., Fernández-López, J., & Pérez-Álvarez, J.A. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour and Fragrance Journal*, *25*, 13-19.

6 Capacidade antioxidante, toxicidade e genotoxicidade *ex vivo* de araçá e butiá liofilizados.

Com base nos resultados obtidos no trabalho apresentado no item 6, que evidencia o maior potencial antioxidante do araçá, quando comparado ao butiá e de que este, especialmente a variedade amarela, possui maior distribuição regional, estes frutos foram selecionados para os experimentos *in vivo.* Neste capítulo são apresentados os resultados obtidos da avaliação dos efeitos de um tratamento sub-crônico a partir do consumo dos frutos por camundongos. O manuscrito científico foi submetido ao periódico *Toxicology Reports,* ISSN: 2214-7500.

Assessment of toxicity, genotoxic and antioxidant effect of *Psidium cattleianum* and *Butia odorata* fruits

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Abstract

The effect of diet supplementation of mice with lyophilized *Butia odorata* (butiá) and *Psidium cattleianum* (araçá) fruits against toxicity and oxidative stress parameters was assessed. Seven groups, with ten mice each, were fed a normal diet and a suspension of 100, 500, and 1000 mg/kg b.w. of lyophilized butiá and araçá fruits or carboxymetilcelulose 0.25% (control) for 35 days. A significant decrease of liver and kidney lipid peroxidation in animals treated with butiá fruits and an increase in the serum antioxidant potential in animals treated with low dose of araçá (100 mg/kg) were observed. A reduction in the lipid peroxidation levels of liver and kidney after butiá consumption indicates direct antioxidant action, while the araçá diet induced an increasing in the plasma antioxidant potential in mice. Both fruits didn't alter the any other biochemical parameter on liver, kidney and brain of mice. These findings support the conclusion that both fruits didn't have any toxic effect and butiá can potentially provide better protection against oxidative stress compared to araçá.
Key words: *Butia odorata*, *Psidium cattleianum*, *ex vivo* antioxidant, genotoxicity.

1. Introduction

Fruits, vegetables and other foods are good sources of several bioactive compounds with antioxidant potential (Dasgupta and Klein, 2014) which can help to maintain human health and delay diseases, such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Shema-Didi et al., 2012; Hofe et al., 2014). The protective effects of these foods result from the presence of vitamins, phenolics, carotenoids, flavonoids and anthocyanins, but also may be the result of unknown compounds or synergy of several antioxidants (Harasym and Oledzki, 2014).

In this context, the protective effect of natural antioxidants in human body is universally accepted. On the other hand, well-known natural antioxidants as vitamin C, vitamin E, carotenoids and polyphenols, may exert toxic pro-oxidant activity (Rietjens et al., 2002), leading to DNA, protein and lipid damage (Galati and O'Brien, 2004).

Tropical countries have a large amount of native and exotic fruit species, which are regionally consumed and have been received special attention by their nutritional and biological values (Jacques et al., 2009; Clerici and Carvalho-Silva, 2011). *Psidium cattleianum* fruit, also known as strawberry guava and araçá, and *Butia odorata*, known as jelly palm or butiá, are cultivated in South America (Henderson et al., 1995). There are two cultivars of araçá, red and yellow fruits, and the chemical composition and biological properties of them have been evidenced. It comprises *in vitro* antioxidant, antimicrobial, antiproliferative and anti-inflammatory capacities of their extracts (Luximon-Ramma et al., 2003; Fetter et al., 2010; Medina et al., 2011; McCook-Russell et al., 2012).

B. odorata fruits are widely consumed in natura or as raw material to make juice, jams and liquor in southern region of America. However, few studies reported their chemical composition and biological properties. Considering the nutritional relevance of *Butia* species, *B. capitata* fruits has significant carotenoids content, pro-vitamin A and essential fatty acids (Lopes et al., 2011; Ferrão et al., 2013). According The American Institute of Medicine, 100g of *B. capitata* pulp fruit can supplement 100% of vitamin A needed in human diet (IOM, 2001). *B. capitata* fruits also have a strong *in vitro* antioxidant capacity when compared to other native fruits, wich can be related to the carotenoid content (Pereira et al., 2013).

The *in vitro* antioxidant capacity is very specific and not necessarily reflects the normal biological context in which the fruits are consumed. According, the *in vivo* health benefits of the consumption of antioxidant-rich fruits as araçá and butiá was not elucidated. Thus, the present study was designed to investigate the antioxidant potential and toxicity of *P. cattleianum* and *Butia odorata* fruits on subchronic treatment in mice. Biochemical variables (aspartate aminotransferase, alanine aminotransferase, urea, creatinine and glucose serum levels) as well as markers of oxidative stress, including the determination of lipid peroxidation (TBARS), catalase (CAT) and δ-aminolevulinate dehydratase (δ-ALA-D) activities, *L*-ascorbic acid, non-protein thiol levels and serum ferric reducing antioxidant power were evaluated. The genotoxicity of treatments was evaluated by comet assay of animal's blood cells.

2. Materials and Methods

2.1. Plant material

Red araçá (*P. cattleianum*) and yellow butiá (*B. odorata*), the fruits used in this study, were obtained from Embrapa Clima Temperado (Pelotas/RS/Brazil) and in the rural area of the municipality of Tapes (RS/Brazil), respectively. The period of collect was between February and March 2012. Fruits were washed and the pulp, along with peel, was manually separated from the seeds and then was lyophilized. Samples were storage at -80 °C until analysis.

2.2. Chemical composition of samples

2.2.1. Phenolic compounds

Total phenolic content was determined using the method described by Singleton and Rossi (1965). The results were expressed in mg of gallic acid per 100 g of sample.

2.2.2. Anthocyanins

The total anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalents per 100 g of sample according Lees and Francis (1972).

2.2.3. Carotenes

Total carotene content was determined using the equations described by Rodrigues-Amaya (2001) and expressed as mg of β-carotene per 100 g of sample.

2.2.4. L-ascorbic acid

L-ascorbic acid was determined using the method described by Vinci, Rot & Mele (1995) and by Ayhan et al. 2001. HPLC Shimadzu system using a reverse phase Shimadzu (Kyoto, Japan) Shim-Pak CLC-ODS column (3.9 cm x 150 mm x 4 µm) was used. Identification was based on retention time comparison to an *L*-ascorbic acid standard (Synth, Diadema, SP, Brazil). Quantification was based on an external standard calibration curve and results were expressed as mg of *L*-ascorbic acid per 100 g of sample.

2.3. Animals and Treatment

Healthy male Swiss albino mice, with an average body weight 35 ± 0.5 g and aged 7-8 weeks old, were obtained from the Central Biotherium of Federal University of Pelotas (Brazil). The Committee on Care and Use of Experimental Animal Resources, the Federal University of Pelotas, Brazil approved the experimental protocols for this study. Mice were housed in metabolic cages (one animal/cage) under standard room temperature (23 ± 2 °C) and 12 h light/dark cycle. To these mice were given standard food and fresh water *ad libitum* during the first 5 days to acclimatization. The animals were divided in 7 groups of 10 animals for each treatment. These groups were used for both biochemical analysis and genotoxicity assay (comet assay). Group 1: Control received water solution of carboxymethyl cellulose (CMC) 0.25%; Group 2: araçá 100 mg/kg body weight (b.w.); Group 3: araçá 500 mg/kg b.w.; Group 4: araçá 1 g/kg b.w.; Group 5: butiá 100 mg/kg b.w.; Group 6: butiá 500 mg/kg b.w.; Group 7: butiá 1 g/kg b.w. Samples of lyophilized fruits were prepared using water distilled with CMC 0.25%.

Doses of *P. cattleianum* and *B. odorata* lyophilized fruits were made available to mice by drinking fountain once daily during 5 hours (9 am - 2 pm) for 35 consecutive days. To the control group of DNA damage induced by methyl methanesulfonate (MMS) for the comet assay, a separate group of animals received CMC (0.25%) and 24 h before the euthanasia, they received a single dose of MMS (200 mg/kg) by intraperitoneal (i.p.) injection. After 35 days the body weight gain was measured and Food Efficiency Coefficient (FEC) was calculated using the equation below:

$$\text{FEC} = \text{weight gain (g)} / \text{total consumed food (g)}$$

2.4. Biochemicals measurement

After 35 days of treatment, mice were euthanized with anesthesia followed by cervical dislocation and blood, liver, kidney and brain were rapidly removed. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (brain: 1/5 w/v; liver and kidney: 1/10 w/v). The homogenate was centrifuged for 10 min at 2000 g to yield a pellet that was discarded, and a low-speed supernatant (S_1) was used for assays.

2.4.1. Hepatic, renal function and glucose serum level

Blood samples were allowed to stand at room temperature for 30 min and serum was isolated by centrifugation at 1000 g for 15 min and used for estimation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, creatinine, urea and glucose levels using standard kits for colorimetric assays (Labtest, MG - Brazil).

2.4.2. Enzymatic activity and non-enzymatic endogenous antioxidant system

δ-Aminolevulinate dehydratase (δ-ALA-D) activity δ-ALA-D is a sulfhydryl-containing enzyme that can be inhibited in different pro-oxidant situations and can be used as a marker of toxicity (Folmer et al., 2003). δ-ALA-D activity was assayed according to the method described by Sassa (1982) by measuring the rate of product (porphobilinogen) formation, with the following modification: 84 mM potassium phosphate buffer (pH 6.4) and 2.5 mM δ-aminolevulinic acid were used. All experiments were carried out after 10 min of pre-incubation of 100 µl of S_1, meaning that the reaction was started by adding the substrate (aminolevulinic acid). Incubations were carried out for 30 min for liver, 1 h for kidney and 3 h for brain at 37 °C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×104 cm^{-1} M^{-1} for the Ehrlich-porphobilinogen salt. Catalase activity (CAT), an enzymatic antioxidant defense, was assayed spectrophotometrically using the method of Aebi (1984), which monitors the disappearance of H_2O_2 in the presence of S_1 at 240 nm. CAT catalyzes the formation of oxygen and water from two molecules of H_2O_2. The presence of ascorbic acid and non-protein thiol groups in tissues, non-enzymatic defenses, was determined as described by Jacques-Silva et al. (2001) and Ellman (1959), respectively. Protein content tissues was measured spectrophotometrically at 650 nm using the method described by Lowry et al. (1951).

2.4.3. Lipid peroxidation

Lipid peroxidation of liver, kidney and brain was estimated by the measurement of thiobarbituric acid reactive species (TBARS), where malondialdehyde (MDA) is one of the end-products of lipid peroxidation detected.

TBARS was determined spectrophotometrically as previously described by Ohkawa et al. (1979).

2.4.4. Determination of antioxidant status of plasma by ferric reducing ability (FRAP) assay

Plasma antioxidant status was evaluated using ferric reducing antioxidant power (FRAP) assay. The assay was carried out according to the method of Benzie and Strain (1996). Plasma (10 µL) was mixed with 990 µL of FRAP reagent and were placed at 37 ºC in a water bath and the absorbance was measured after incubation time at 593 nm.

2.5. Evaluation of genotoxicity effect - Comet assay

The alkaline comet assay was performed as described by Collins (2004), with modifications. Peripheral blood of mice was collected by cardiac puncture after 35 days of treatment with fruits. An aliquot of blood sample was mixed with low-melting point agarose (10 µL of blood sample was added to 90 µL of LMP agarose 0.75% w/v) and placed on a microscope slide pre-coated with normal melting point agarose 1% (w/v). A cover slip (100 µL) was added and after agarose solidified, cover slips were removed, and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0-10.5) containing 1% Triton X-100 and 10% DMSO. The slides were kept frozen in lysis solution (4 ºC) and protected from the light for approximately 14 h. They were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13.5) for 20 min for DNA unwinding. Electrophoresis (20 min at 300 mA and 25 V) was performed in the same buffer. Every step was carried out under indirect yellow light. After electrophoresis, the slides were neutralized in Tris 400 mM (pH 7.5), rinsed three times in distilled water, and left to dry overnight at room temperature. Dry slides were re-hydrated for 3 min with distilled water and were then fixed for 10 min in trichloroacetic acid 15% (w/v), zinc sulfate 5% (w/v) and glycerol 5% (v/v), rinsed three times in distilled water, and dried for at least 5 h. Dry slides were re-hydrated for 3 min with distilled water, stained [sodium carbonate 5% (w/v), ammonium nitrate 0.1% (w/v), silver nitrate 0.1% (w/v), tungstosilicic acid 0.25%, formaldehyde 0.15% (w/v), freshly prepared in the dark], and constantly shaken for 25 min. Slides submerged in the stop solution (acetic acid

1%) were rinsed again and immediately tagged for analysis. One hundred randomly selected cells per sample were scored visually according to tail intensity into five classes (from undamaged, 0, to maximum damaged, 4).The damage index (DI) was based on the length of migration and on the amount of DNA in the tail and was calculated as follows:

$$DI = n1 + 2n2 + 3n3 + 4n4,$$

where n1 represents the number of cells with level 1 of damage; n2, number of cells with level 2 of damage; n3, number of cells with level 3 of damage; and n4, number of cells with level 4 of damage.

2.6. Statistical analysis

Results of all biochemical analysis were expressed as the mean ± standard deviation (SD). Results of genotoxicity (comet assay) were expressed as the mean ± standard error (SE) and analysed by Kruskal-Wallis one-way analysis of variance (ANOVA) . All of other data were validated using one-way analysis of variance followed by a Newman-Keuls test when appropriate ($p < 0.05$).

3. **Results and discussion**

3.1. Chemical composition

Both fruits had difference in total phenolic, carotenoids, anthocyanins and *L*-ascorbic acid content. Araçá showed more phenolic content (875.43 ± 7.44 mg gallic acid/100 g d.w.) than butiá (864.42 ± 7.13 mg gallic acid/100g d.w.) ($p < 0.05$) and around 21% more anthocyanins (143.17 ± 4.76 mg cyanidin-3-glucoside/100 g d.w.) content than butiá (126.25 ± 6.69 mg cyanidin-3-glucoside/100 g d.w.) ($p < 0.01$). Butiá carotenoids content (74.09 ± 1.39 µg lycopene/100 g d.w.) was 2.5 times higher than araçá (29.63 ± 1.75 µg lycopene/100 g d.w.) ($p < 0.001$). *L*-ascorbic acid (77.45 ± 4.08 mg/100g d.w.) concentration also was higher than in araçá (16.31 ± 0.12 mg/100g d.w.) ($p < 0.001$). Carotenoids have an important role in human diet because they are precursor of vitamin A and have antioxidant properties as singlet oxygen quenching and free radicals scavengers (Paiva and Russell, 1999). In this line, the carotenoids content and *in vitro* antioxidant capacity of *B. capitata* fruits were described by Pereira et al. (2013). These features extend to understanding of the

nutraceutical importance of butiá as a dietary source of β-carotene and pro Vitamin A (Faria et al., 2011).

3.2. Body weight gain and food efficiency coefficient (FEC)

The body weight gain of mice were checked and are shown in Table 1. After 35 days, the intake of araçá, at 1 g/kg b.w. and butiá at 100 mg/kg and 1 g/kg b.w., increased the body weight of mice without changes in food efficiency coefficient (FEC). FEC was equivalent for all groups indicating apparently no changes in the metabolism of carbohydrates, protein and lipid due fruits intake. On the other hand, the increase in body weight could be related to the intake of carbohydrate, protein and lipid from both fruits.

Table 1 - Body weight gain and food efficiency coefficient (FEC) of mice submitted to diet with *P. cattleianum* and *B. odorata* lyophilized fruits during 35 days.

Group	Body weight gain (g)	FEC
Control	2.62 ± 1.26	0.017 ± 0.006
Araçá 100 mg/kg	4.28 ± 2.04	0.014 ± 0.008
Araçá 500 mg/kg	3.66 ± 1.85	0.013 ± 0.003
Araçá 1000 mg/kg	5.87 ± 1.10 *	0.014 ± 0.009
Butiá 100 mg/kg	5.12 ± 1.23 *	0.013 ± 0.008
Butiá 500 mg/kg	2.52 ± 1.72	0.001 ± 0.010
Butiá 1000 mg/kg	5.16 ± 2.22 *	0.016 ± 0.005

Results were expressed as the mean ± standard deviation (SD). (*) $p < 0.01$ as compared to control group (CMC 0.25%). One-way ANOVA followed by the Student-Newman Keuls test was used for comparison.

3.3. *Biochemical measurement*

3.3.1 AST and ALT activity, urea, creatinine and glucose levels

The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes linked to amino acid metabolism. Their activities are biochemical markers for liver function and integrity. Thus, elevation in the serum activities of both enzymes is commonly associated with leakage from hepatocytes and possible damage, which might have resulted from change in membrane permeability (Latha et al., 1998). The serum levels of urea and creatinine have been used as a biomarker to detect toxic effects on the kidney (Davis and Bredt, 1994). Urea is the major nitrogen containing metabolite of protein metabolism and creatinine is endogenously produced and released into body fluids, used as an indicator of glomerular filtration rate (Burtis and Ashwood, 1996). The effects of doses of araçá and butiá on selected liver and renal functions, in biochemical parameters and glucose levels in serum are presented in Table 2. Treatment of mice at all doses investigated of both fruits after 35 days did not alter these parameters in comparison to the control group.

Table 2 – Serum analysis of mice after intake of *P. cattleianum* and *B. odorata* lyophilized fruits (100-1000 mg/kg b.w.).

Group (mg/kg)	**AST (U/ml)**	**ALT (U/ml)**	**Urea (mg/dL)**	**Creatinine (mg/dL)**	**Glucose (mg/dL)**
Control	36.08 ± 7.1	139.20 ± 32.2	39.27 ± 10.8	0.34 ± 0.1	131.30 ± 10.1
Araçá 100	44.83 ± 5.8	151.30 ± 32.5	50.58 ± 4.8	0.33 ± 0.0	138.60 ± 24.8
Araçá 500	38.33 ± 11.2	155.30 ± 20.1	33.65 ± 6.9	0.36 ± 0.0	127.70 ± 27.5
Araçá 1000	45.00 ± 3.0	142.00 ± 28.7	55.08 ± 12.5	0.40 ± 0.0	144.90 ± 23.8
Butiá 100	32.60 ± 2.9	132.20 ±28.0	39.04 ± 10.8	0.37 ± 0.0	123.31 ± 20.1
Butiá 500	38.10 ± 8.0	119.30 ± 17.2	37.39 ± 18.3	0.36 ± 0.0	138.30 ± 27.3
Butiá 1000	36.30 ± 4.0	131.30 ± 15.7	31.65 ± 8.3	0.39 ± 0.0	137.20 ±35.2

Results were expressed as the mean ± standard deviation (SD).

3.3.1. Ascorbic acid and SH-non proteic levels

Glutathione (*L*-γ-glutamyl-L-cysteinyl glycine, GSH) is the most abundant cellular antioxidant of body wish maintains the intracellular redox balance. The levels of GSH in liver and kidney of mice were measured as non-protein thiols (NPSH). Pro-oxidants effects can be linked to decrease of endogenous antioxidants levels as NPSH.

For human, ascorbic acid is an exogenous antioxidant, which depends entirely of diet. In contrast, mice tissues generally have high levels of ascorbic acid, which are only slightly influenced by exogenous vitamin C. NPSH and ascorbic acid levels in liver and kidney were not altered by treatment with all doses of fruits, as shown in Table 3.

Table 3 - Ascorbic acid and thiol-non protein (NPSH) levels on liver and kidney of mice after diet with *P. cattleianum* and *B. odorata* lyophilized fruits (100-1000 mg/kg b.w.).

Group	Ascorbic acid		NPSH	
	Liver	Kidney	Liver	Kidney
Control	283.40 ± 53.1	182.80 ± 21.2	11.11 ± 0.9	8.93 ± 0.4
Araçá 100 mg/kg	269.80 ± 54.7	184.60 ± 12.3	11.01 ± 0.4	8.82 ± 0.5
Araçá 500 mg/kg	270.90 ± 61.4	193.80 ± 18.3	10.41 ± 1.3	9.19 ± 0.7
Araçá 1000 mg/kg	248.60 ± 54.8	175.30 ± 35.9	10.41 ± 1.3	9.25 ± 0.3
Butiá 100 mg/kg	266.80 ± 58.7	183.50 ± 26.3	10.48 ± 0.0	8.86 ± 11.0
Butiá 500 mg/kg	258.90 ± 50.4	186.90 ± 16.9	11.25 ± 1.0	9.54 ± 0.6
Butiá 1000 mg/kg	276.20 ± 53.6	188.00 ± 20.3	10.75 ± 1.1	9.08 ± 0.3

Results were expressed as the mean ± standard deviation (SD) of ascorbic acid (µg/g tissue) and NPSH (µmol/g tissue) levels in liver and kidney of mice.

3.3.3 *Enzymatic activity - CAT and δ-ALA-D*

Catalases are heme-containing enzymes that convert hydrogen peroxide (H_2O_2) in water and O_2. They are confined in organelles such as peroxisomes, mitochondria and the endoplasmic reticulum. The dismutation of H_2O_2 is an important reaction of antioxidant system defense (Halliwell and Gutteridge, 1999). The CAT

activity of mice did not alter after treatment with all doses of fruits, as can see in Fig. 1. Taken together, treatments for the period of experiment did not significantly affect levels of endogenous antioxidants (ascorbic acid and NPSH) and CAT activity. It can be seen that butiá and araçá at doses of 100-1000 mg/kg b.w. did not show any pro-oxidant effect in liver, kidney and brain of mice.

We evalueted the effect of fruits in the δ-ALA-D activity in liver, kidney and brain of mice (Table 4). This enzyme displays an important role in biosynthesis of group heme of hemoglobin by catalyzing the condensation reaction of two molecules of δ-aminolevulinic acid to form porphobilinogen (a precursor of heme, cytochromes and other heme-proteins). The oxidation of sulfhydryl group of the active site of δ-ALA-D, changes the configuration of the enzyme and impairs its activity. Indeed, the impairment of δ-ALA-D functionality may impair heme biosynthesis and result in the accumulation of δ-aminolevulinic acid (ALA). The accumulation of ALA affects the aerobic metabolism and causes some pro-oxidant activity. In this context, we assume that the intake of fruits, at all doses studied, did not cause pro-oxidant effects linked to inhibition of δ-ALA-D activity after 35 days.

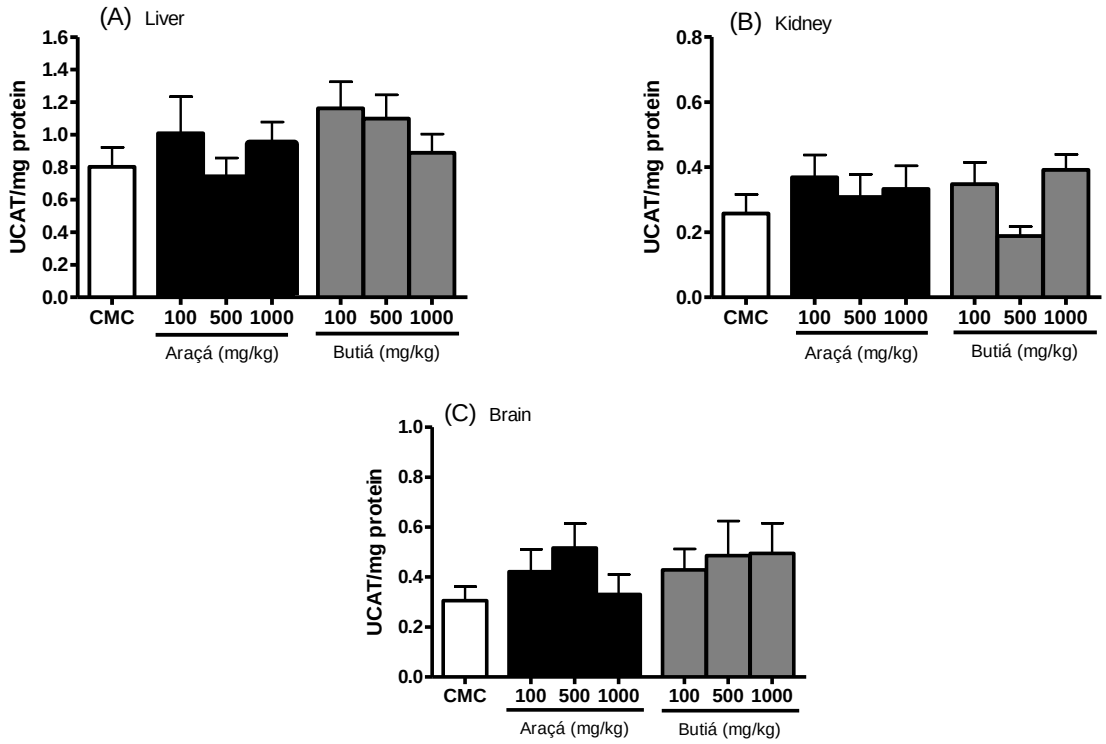

Figure 1 - CAT activity in (A) liver, (B) kidney and (C) brain of mice after subchronic treatment with *P. cattleianum* and *B. odorata* lyophilized fruits.

Table 4 - δ-ALA-D activity of liver, kidney and brain of mice after sub-chronic treatment with different doses (100-1000 mg/kg b.w.) of *P. cattleianum* and *B. odorata* lyophilized fruits.

Group	Liver	Kidney	Brain
Control (CMC)	10.96 ± 3.31	1.84 ± 0.34	1.62 ± 0.44
Araçá 100 mg/kg	11.07 ± 3.06	1.84 ± 0.52	1.62 ± 0.43
Araçá 500 mg/kg	10.26 ± 3.24	2.15 ±0.62	1.79 ± 1.07
Araçá 1000 mg/kg	12.55 ± 1.45	1.88 ± 0.40	1.74 ± 0.68
Butiá 100 mg/kg	12.36 ± 2.21	1.78 ± 0.37	1.84 ± 0.90
Butiá 500 mg/kg	12.94 ± 2.85	2.23 ± 0.40	1.89 ± 0.75
Butiá 1000 mg/kg	12.04 ± 3.15	1.99 ± 0.49	1.64 ± 0.47

Results were expressed as the mean ± standard deviation (SD) of nmol PBG/mg protein/h.

3.3.4 *Lipid peroxidation*

Lipids are the major components of cell membrane and lipid peroxidation is one of the most important consequences of oxidative stress, involved in apoptosis and diseases progression (Reiter et al., 1998; Spector, 2000; Dasgupta and Klein, 2014).

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are end-products of lipid peroxidation, have cytotoxic and genotoxicity effects in normal cell and they are found in higher levels in some pathologies, such as cancer (Cai et al., 2012). Additionally, accumulation of MDA in tissues or biological fluids is an indicative of the extent of free radical generation, oxidative stress and tissue damage (Gutteridge, 1995).

According data presented in Fig. 2, no significant difference was observed in TBARS levels in liver, kidney and brain of mice treated with all doses of araçá when compared to the control group.

On the other hand, the treatment with butiá at dose of 100 mg/kg showed approximately 37% of decreased in lipid peroxidation of liver ($p < 0.05$). The doses of 500 and 1000 mg/kg also showed decreased of 42% and 45%, respectively in lipid peroxidation of kidney when compared to the control group ($p < 0.05$). The basal lipid

peroxidation in brain was not altered by butiá doses. Exogenous antioxidant compounds as vitamins C and E, carotenoids and polyphenols have antioxidant activity against excess ROS generated during normal cellular metabolism, as mitochondrial respiration. More specifically, Vitamin C prevents lipid peroxidation processes (Valko et al., 2007). The observed effects of decreasing in lipid peroxidation in liver and kidney induced by treatment with butiá can be explained in part by the high concentrations of carotenoids and ascorbic acid in *B. odorata* when compared to *P. Cattleianum* fruits.

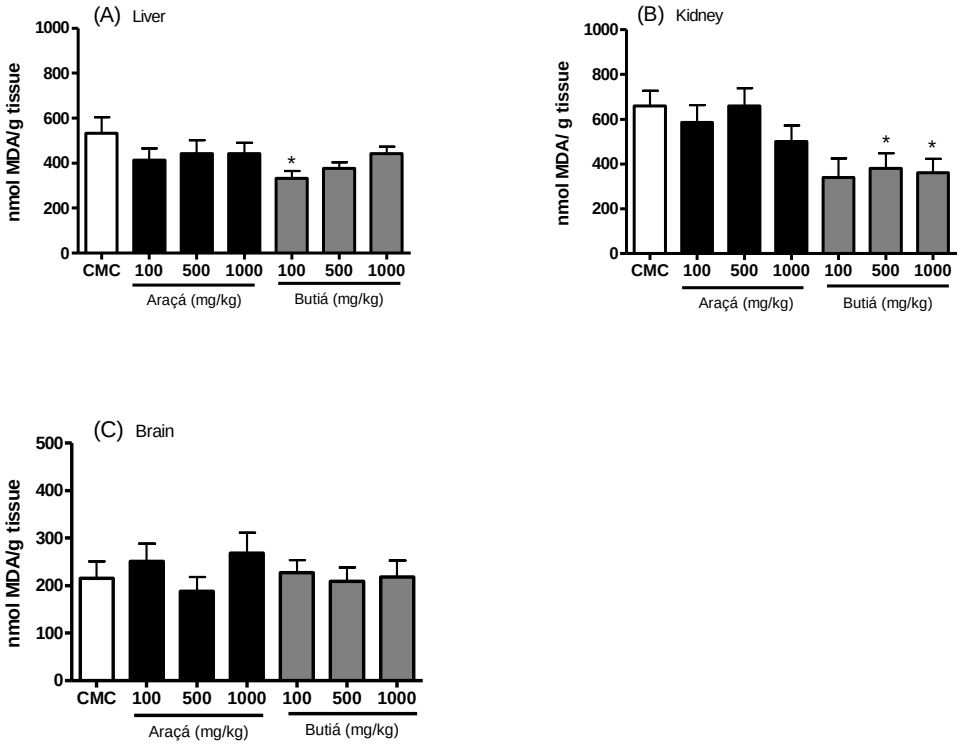

Figure 2 - Lipid peroxidation of (A) liver, (B) kidney and (C) brain of mice after subchronic treatment with *P. cattleianum* and *B. odorata* lyophilized fruits. (*) $p < 0.05$ when compared to control group (CMC).

3.3.5 Serum *antioxidant status*

Total antioxidant capacity in blood serum was used as a biomarker to access the antioxidant status because all antioxidants present in a sample can alter this parameter. The antioxidant status in serum was estimated by measuring the increase in absorbance at 593 nm caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2,4,6-tripyridyl-s-triazine) and $FeCl_3.6H_2O$. As shown in Fig. 3, only

the dose of 100 mg/kg of red araçá increased the antioxidant status in blood serum of mice. On the other hand, butiá did not alter this parameter in all the tested doses.

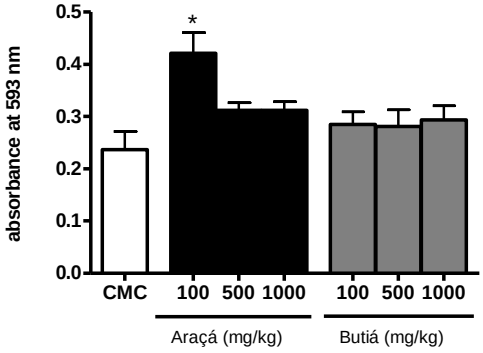

Figure 3 - Serum antioxidant status of mice after sub-chronic treatment with *P. cattleianum* and *B. odorata* lyophilized fruits. (*) $p < 0.05$ when compared to control group (CMC).

3.4. Genotoxicity of subchronic treatment - Comet assay

The comet assay or single cell gel electrophoresis is a widely used technique for measuring and analyzing DNA breakage in individual cells, which can be appropriated to evaluate *in vivo*, *ex vivo* and *in vitro* effects. Comet assay is a sensitive and valuable technique to observe cytogenetic damage and it reveals prooxidant and antioxidant effects of endogenous and exogenous agents (Anderson & Phillips, 1999). The antioxidant status of blood serum is directly linked to diet and is attributed to components such as vitamins, phenolic compounds and carotenoids. It was in the comet assay primary single (repairable damage), double-strand DNA breaks and alkali-labile sites in the alkaline version. Several studies show the *ex vivo* antigenotoxic capacity of fruits extracts, where they showed any prooxidant effects and DNA damage (Malta et al., 2012; Neri-Numa et al., 2013).

By the first time, our work showed the impact of treatment of mice with *P. cattleianum* and *B. odorata* fruits in DNA integrity of blood cells of mice. As can see in Table 5, treatment of animals with all doses of both fruits did not alter DNA integrity of mice when compared to the control group (CMC 0.25%). On the other hand, as

expected, animals treated with MMS (200 mg/kg, i.p.) had a significant increase in DNA damage induced by methylation reactions.

Our results are in agreement of some studies that showed that the consumption of a controlled diet hight in fruit-juice (apple, mango and orange juice) for 14 days didn't change the total antioxidant capacity, decreased the basal lipid peroxidation in plasma of healthy people and no effect on DNA integrity was observed (Bub et al., 2003). In contrast of our results, another study showed that the consumption of a mix of tropical fruit juices (acerola, açaí, yellow mombin, cashew-apple, camu-camu and pineapple) during 4 weeks by rats caused a significant change in the liver SOD and CAT activities, reducing erythrocytes GPx activity, but also decreased TBARS levels (Pereira et al., 2014).

Table 5 - Distribution of comet classes and DNA damage index in blood peripheral of mice after diet with *P. cattleianum* and *B. odorata* lyophilized fruits (100-1000 mg/kg b.w.).

Group	Comet class (%)					DI mean ± SE
(mg/kg)	**0**	**1**	**2**	**3**	**4**	
Control (CMC)	94.46 ± 1.99 [a]	4.53 ± 1.69 [a]	0.23 ± 0.16 [a]	0 [a]	0 [a]	5.0 ± 1.77
MMS 200	0 [b]	65.25 ± 1.88 [b]	24.25 ± 1.49 [b]	6.75 ± 0.85 [b]	3.75 ± 0.62 [b]	149.0 ± 2.44 [*]
Araçá 100	96.38 ± 1.90 [a]	3.62 ± 1.90 [a]	0 [a]	0 [a]	0 [a]	3.62 ± 1.90
Araçá 500	90.25 ± 4.40 [a]	9.62 ± 4.29 [a]	0.12 ± 0.12 [a]	0 [a]	0 [a]	9.87 ± 4.51
Araçá 1000	94.0 ± 2.16 [a]	5.57 ± 2.20 [a]	0.42 ± 0.29 [a]	0 [a]	0 [a]	6.42 ± 2.15
Butiá 100	91.0 ± 3.86 [a]	9,0 ± 3.86 [a]	0 [a]	0 [a]	0 [a]	9.0 ± 3.86
Butiá 500	93.0 ± 1.93 [a]	6.8 ± 1.87 [a]	0.20 ± 0.13 [a]	0 [a]	0 [a]	7.2 ± 1.99
Butiá 1000	95.10 ± 1.73 [a]	4.90 ± 1.73 [a]	0 [a]	0 [a]	0 [a]	4.90 ± 1.73

Data are expressed as mean ± standard error (SE) of DNA damage index (DI) and DNA damage levels (1-4). Kruskal-Wallis one-way analysis of variance (ANOVA) was used for comparison. Different small letters in the same column denote $p < 0.05$. (*) Denoted $p < 0.001$ when compared to negative control group (CMC 0.25%).

4. Conclusion

The present study evaluated antioxidant and toxicity effects of *P. cattleianum* and *B. odorata* lyophilized fruits intake during 35 days by mice. The antioxidant capacity of butiá was demonstrated by decrease of basal lipid peroxidation in liver and kidney of mice. Araçá showed an indirect antioxidant capacity by an increase of serum antioxidant status. Doses selected of fruits did not cause any changes on biochemical and genotoxic parameters. To the best of our knowledge, this is the first report on the evaluation of antioxidant and genotoxic effects in a sub-chronic treatment with *P. cattleianum* and *B. odorata* fruits.

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7 Considerações finais e perspectivas

Os objetivos propostos para a pesquisa compreendida nesta tese foram atingidos. Conforme esperado e, de acordo com a atividade antioxidante de compostos fenólicos amplamente relatada na literatura, as hidroxibenzalacetonas DHZ, iso-deidrozingerona e 3,4-diidroxibenzalacetona apresentaram atividade antioxidante em diferentes modelos experimentais *in vitro*. A síntese de novas moléculas contendo grupos orgânicos de selênio, a partir da modificação estrutural de hidroxibenzalacetonas apresentou limitação quanto ao rendimento reacional dos produtos finais. No entanto, foi relatada pela primeira vez a síntese de compostos organocalcogênios análogos à DHZ. Dentre estes, os adutos de Michael (**2b** e **2c**) demonstraram potencial antioxidante superior à DHZ quanto a neutralização de radicais DPPH e a capacidade anti-hemólise. Com isso, a hipótese "a" do nosso projeto foi parcialmente comprovada, demonstrando também a importância do grupo OH fenólico associado aos grupos arilcalcogênios na estrutura de compostos análogos à DHZ. Observou-se melhoria na atividade antioxidante relacionada à redução de radicais livres sintéticos, inibição de processos de peroxidação lipídica e hemólise induzida por estresse oxidativo. Com base nesses dados, a síntese de organocalcogênios análogos à DHZ representa uma linha de pesquisa a ser melhor explorada. O seu potencial antioxidante requer estudos complementares quanto aos mecanismos de ação e identificação dos produtos gerados por este efeito e também a realização de ensaios toxicológicos.

Os resultados obtidos a partir do estudo do óleo essencial das folhas da guabirobeira comprovam a hipótese "b", justificada pela presença de compostos terpênicos com atividade antioxidante e antimicrobiana. Além disso, a partir do estudo *ex vivo* foi possível demonstrar que o óleo essencial não alterou os parâmetros bioquímicos de toxicidade avaliados. A partir destes resultados, o presente trabalho elucidou algumas propriedades biológicas ainda não descritas e de óleos essenciais das folhas de *C. xanthocarpa.*

As espécies nativas frutíferas estudadas, especificamente os frutos araçá e butiá apresentam diversos componente funcionais, como a vitamina C, compostos fenólicos, carotenóides e antocianinas, entre outras relatadas na literatura. Neste estudo, a comparação entre os níveis de fitoquímicos denota o maior nível de

compostos fenólicos totais, antocianinas, carotenóides e de ácido *L*-ascórbico do butiá vermelho quando comparado ao butiá amarelo e araçá. Esta diferença não influenciou de forma significativa na capacidade antioxidante *in vitro* demonstrada pelos frutos. Neste sentido, o araçá apresentou maior capacidade em neutralizar radicais livres sintéticos (DPPH, ABTS e $^{\bullet}OH$) e proteger contra a peroxidação lipídica *in vitro* do córtex de camundongos em comparação às duas variedades de butiá estudadas.

Considerando a capacidade antioxidante *in vitro* dos frutos, o maior potencial antioxidante do araçá e a maior distribuição e produção de butiá amarelo na região, os estudos progrediram para a avaliação da toxicidade e potencial antioxidante *ex vivo* destes frutos. A hipótese “c” foi parcialmente provada, pois o butiá demonstrou a capacidade de diminuir os níveis basais de peroxidação lipídica no fígado e no rim de camundongos em diferentes doses. O araçá não apresentou esta capacidade, mas aumentou o potencial redutor do soro sanguíneo dos camundongos tratados com a menor dose (100 mg/kg). A partir dos parâmetros bioquímicos analisados, os tratamentos com ambos frutos não apresentaram efeitos tóxicos e genotóxicos. A partir destes resultados, o presente trabalho traz, pela primeira vez, os efeitos toxicológicos e bioquímicos do consumo de duas frutas nativas, demonstrando como principal efeito do butiá, a dimiuição dos níveis de peroxidação lipídica hepática e renal em camundongos.

Para esclarecimento e aprofundamento dos estudos, sugere-se

- Estudar novos métodos para a síntese de compostos organocalcogênios análogos à zingerona e deidrozingerona, principalmente a fim de ampliar o espectro de compostos orgânicos contendo selênio e enxofre;
- Estudar os mecanismos de ação envolvidos na atividade antioxidante e a toxicidade dos compostos derivados da deidrozingerona (**4d**, **2b** e **2c**) sintetizados neste estudo;
- Estudar a capacidade antioxidante do suco liofilizado de araçá e de butiá em modelos experimentais *in vivo e ex vivo* a fim de avaliar os efeitos de proteção e/ou reversão do estresse oxidativo induzido.

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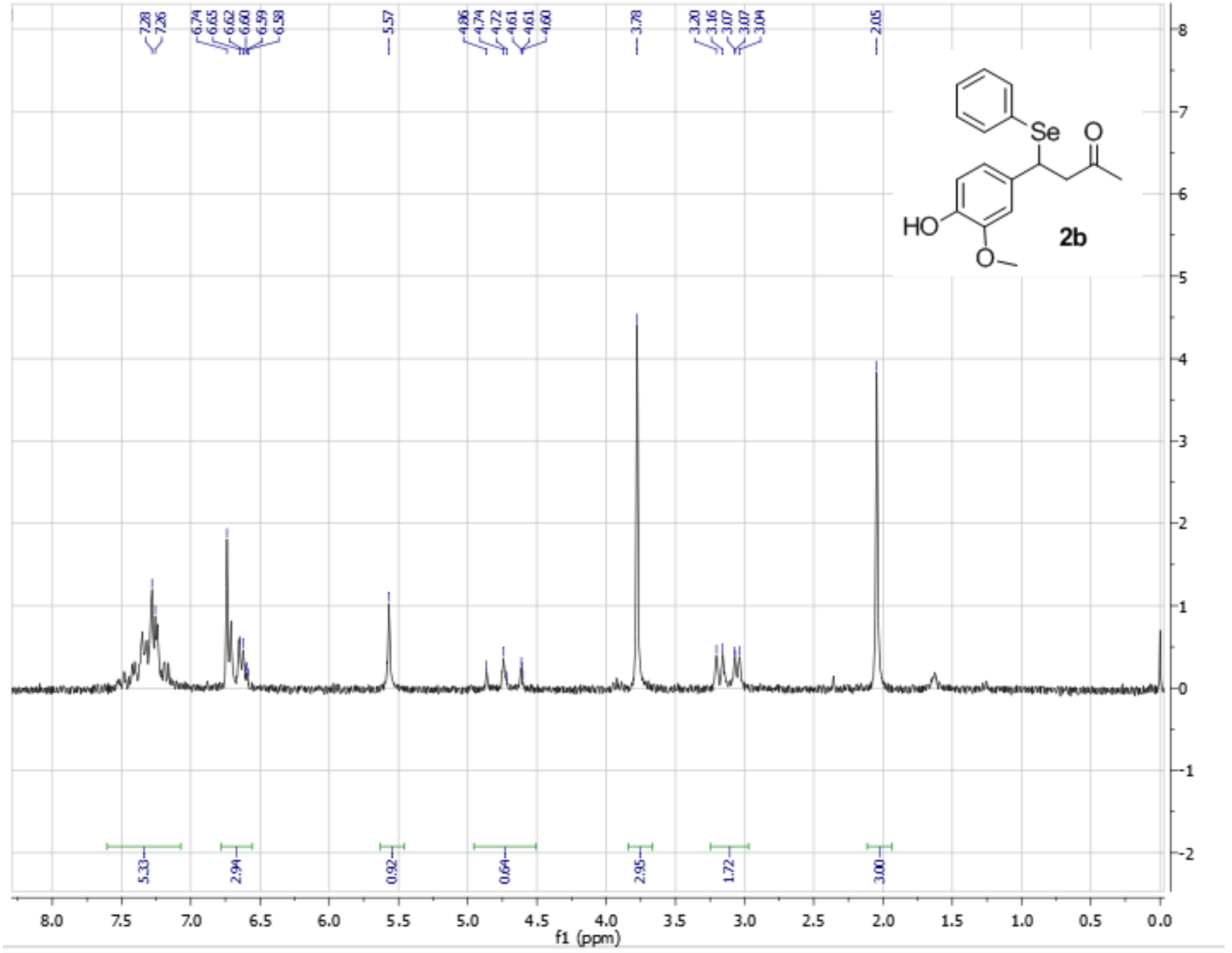
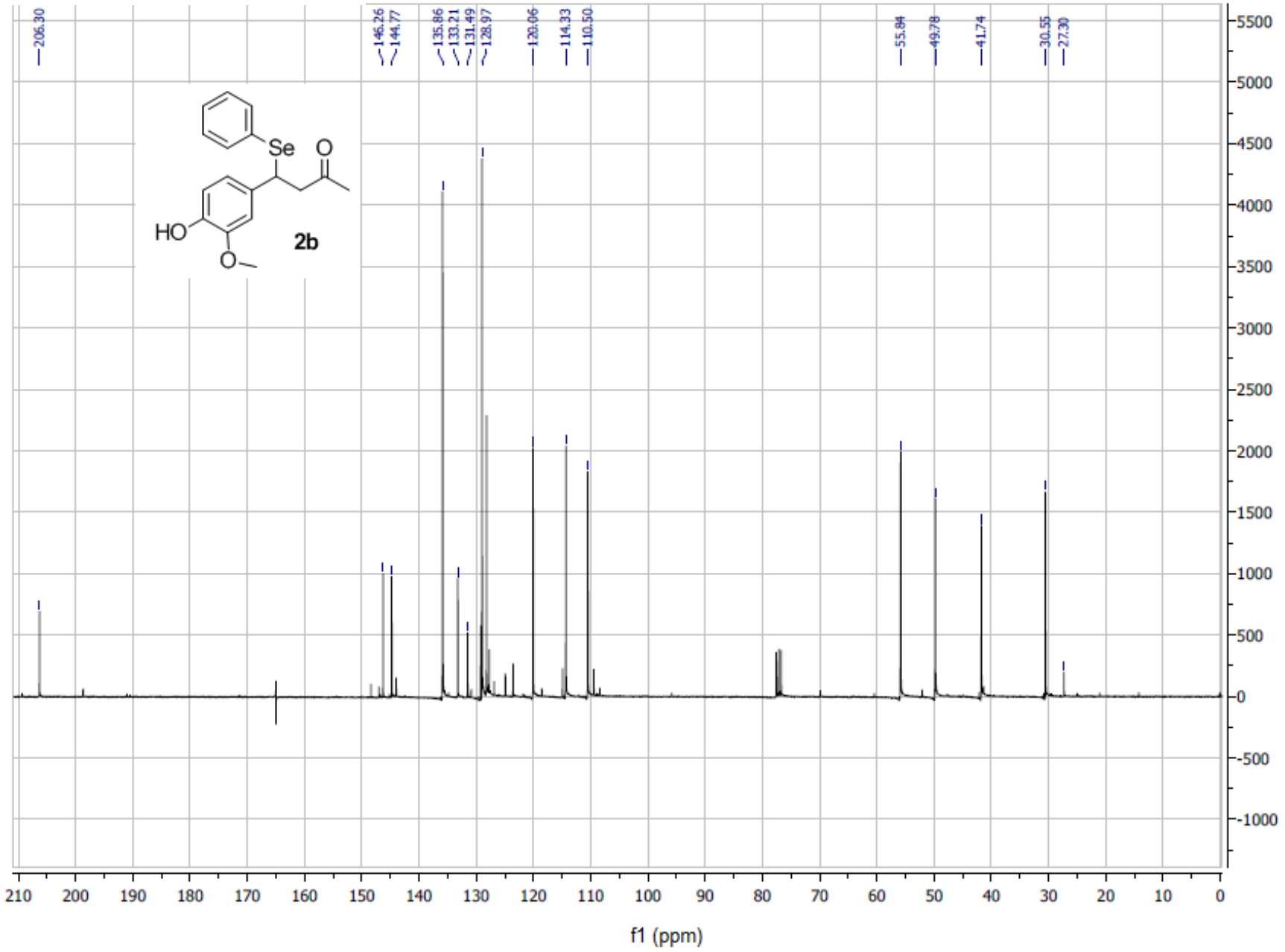
Apêndice A – Espectros de ressonância magnética nuclear (RMN) de ^{1}H e ^{13}C dos compostos 2b, 2c e 4d.

RMN ^{1}H – Composto 2b



RMN ^{13}C – Composto 2b



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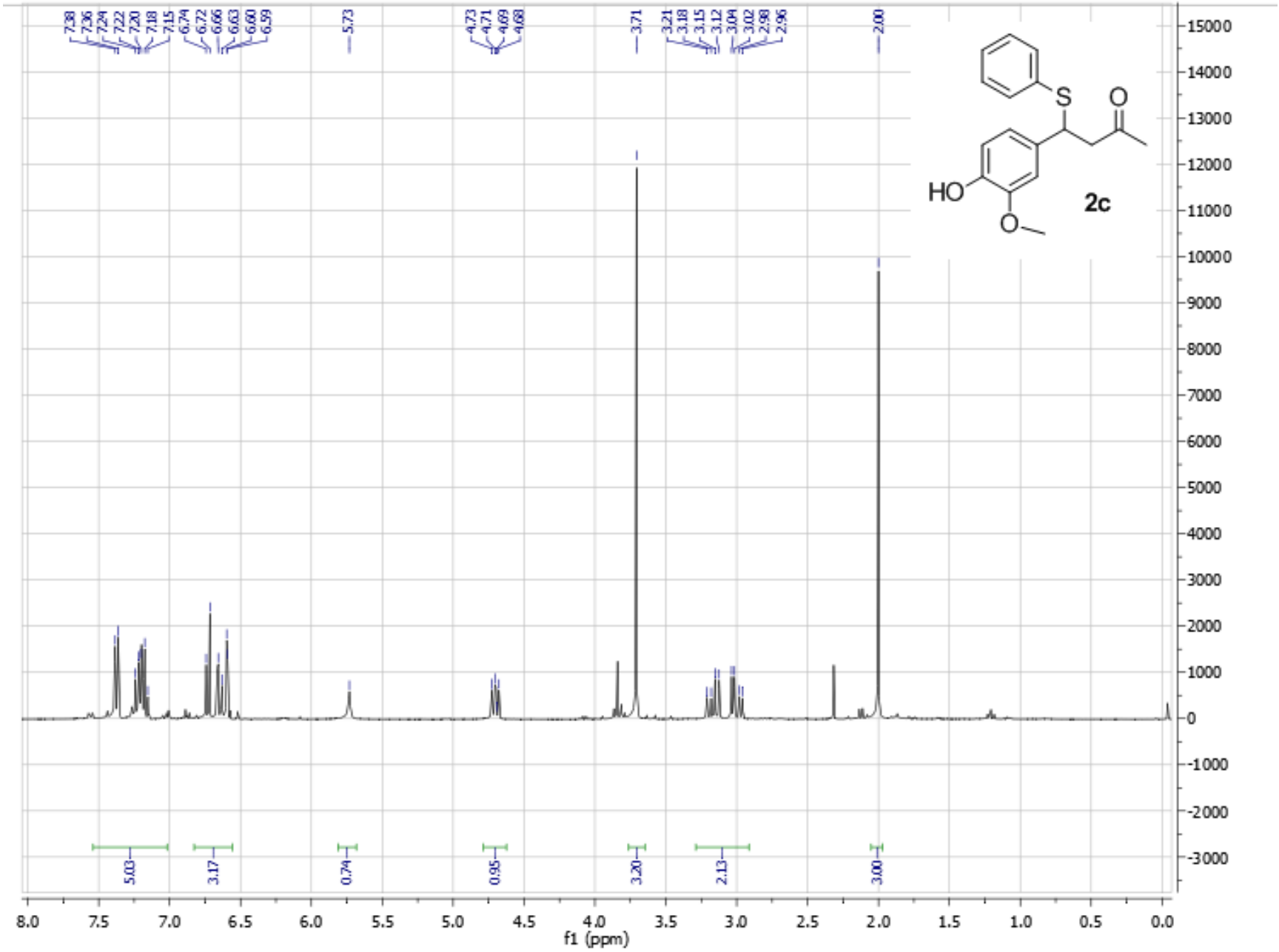
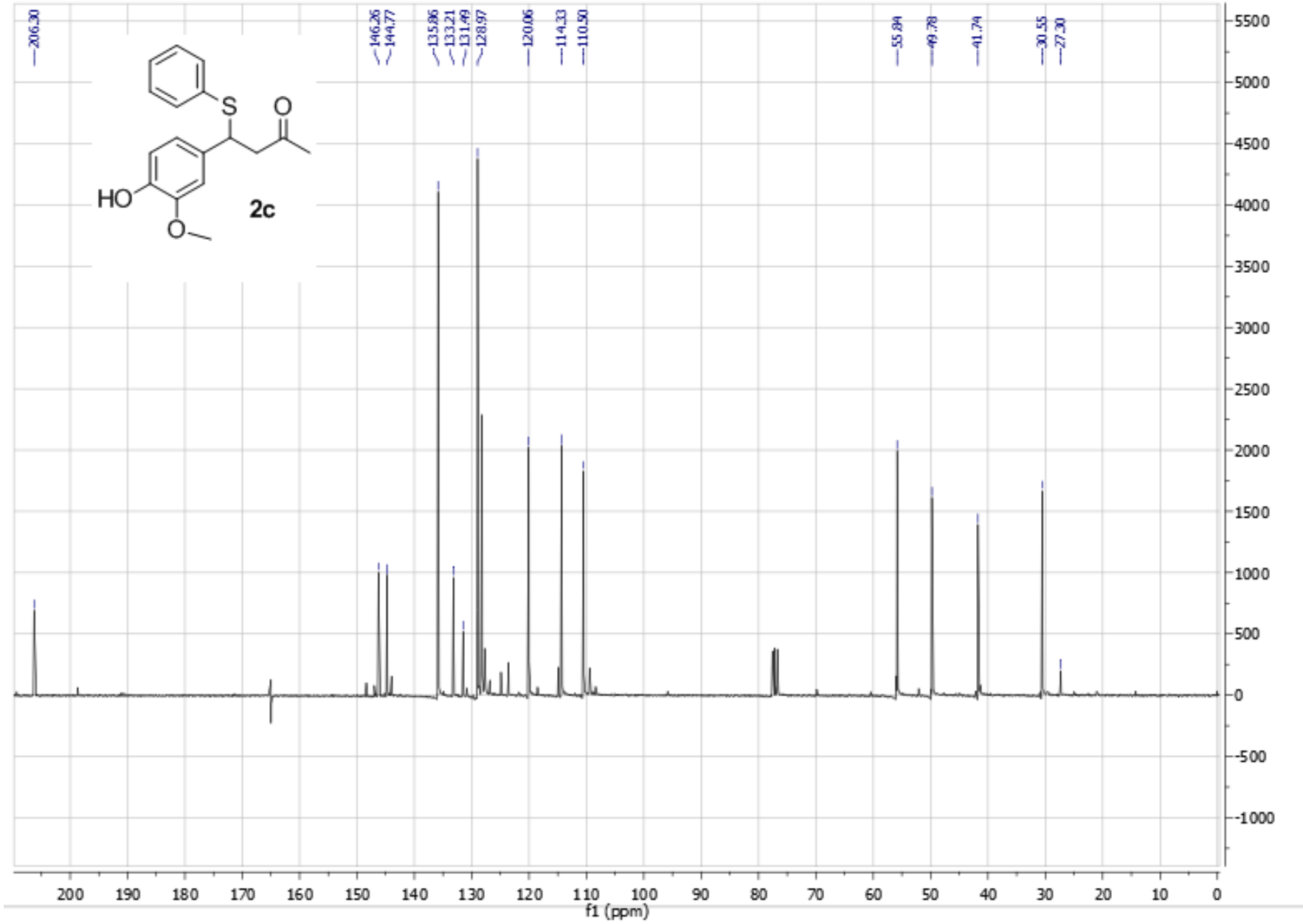
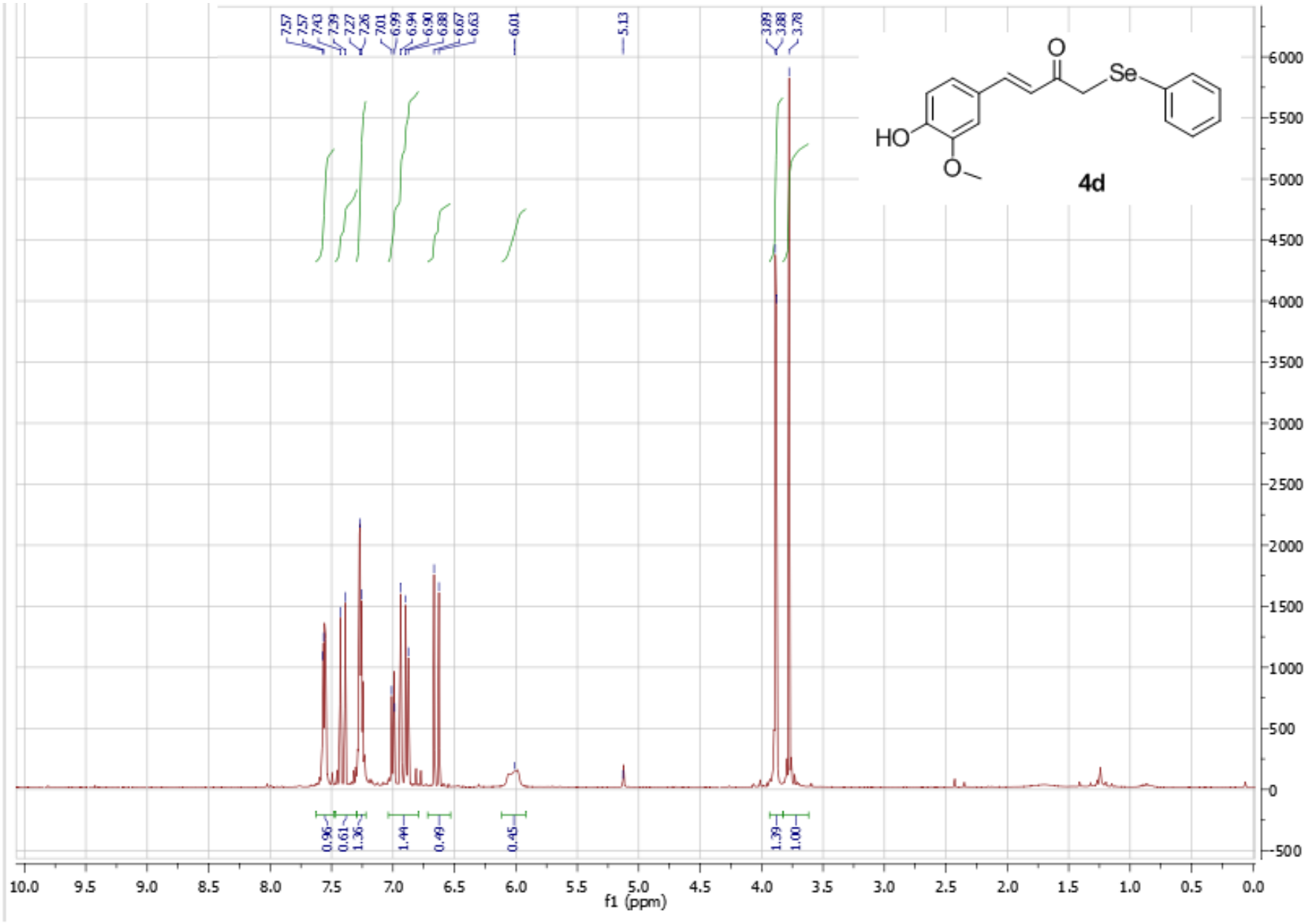


RMN ^{13}C – Composto 2c



RMN ^{1}H – Composto 4d



RMN ^{13}C – Composto 4d